

**Paramoebiasis of sea-farmed salmonids in
Tasmania - a study of its aetiology,
pathogenicity, and control.**



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of Doctor of Philosophy.**

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This thesis contains no material which has been accepted for a degree or diploma by the university or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis.

A handwritten signature in black ink, consisting of a stylized 'L' followed by a large loop.

Teresa Howard
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HOBART
June 2001

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Table of Contents

Acknowledgements	1
Abstract	3
Abbreviations	5

Chapter 1. Background and review of literature

1.1 Sea-farming of salmonids	
1.1.1 The Salmonidae	6
1.1.2 Introduction of salmonids to Australia	7
1.1.3 History of sea-water salmon farming	9
1.1.4 Salmonid farming in Australia	10
1.1.5 Economics of the Tasmanian salmonid industry	13
1.1.6 Sea-farming methods	16
1.2 Major diseases of sea-farmed salmonids	19
1.2.1 Bacterial diseases	20
1.2.2 Viral diseases	26
1.2.3 Diseases caused by protozoans	30
1.2.4 Other diseases	36
1.2.5 Diseases common in Tasmania	38
1.3 Paramoebiasis of aquatic animals	
1.3.1 The amoebae	39
1.3.2 <i>Paramoeba</i> species	41
1.3.3 Diseases of aquatic animals caused by amoebae	51
1.3.4 Paramoebiasis of crabs	58
1.3.5 Paramoebiasis of sea urchins	63
1.3.6 Paramoebiasis of salmonids and turbot	67
1.4 Objectives for this study	80

Chapter 2. Methods

2.1 Culture of marine amoebae	84
2.1.1 Seeding agar plates	85
2.1.2 Subculture	85
2.1.3 Large-scale production	86
2.1.4 Contamination control	87
2.1.5 Revival of failing cultures	88
2.2 Isolation of amoebae from fish gills	
2.2.1 Ammonium chloride wash	88
2.2.2 Cloning of amoebae	89
2.3 Identification of <i>Paramoeba</i> species	
2.3.1 Phase contrast microscopy	90
2.3.2 DAPI-staining	91
2.3.2 Gomori's trichrome stain	91
2.4 Cryopreservation of amoebae	
2.4.1 Cryopreservation	92
2.4.2 Culture retrieval	93
2.5 Antigen preparation and antiserum production	
2.5.1 Antigen Preparation	93
2.5.2 Antiserum production in rabbits	94
2.5.3 Antiserum production in fish	95
2.5.4 Serum preparation	96
2.6 Gill health assessment methods	
2.6.1 Gross examination	97
2.6.2 Preparation of gill tissue for histological examination	97
2.6.3 Immunofluorescence antibody test (IFAT)	98
2.7 Immunostaining techniques	
2.7.1 Immunofluorescence	100
2.7.2 Immunoperoxidase	100
2.7.3 Alkaline phosphatase	101

2.8	Preparation of <i>Paramoeba</i> for electron microscopy	102
2.9	Chemical screening assays	
2.9.1	Preparation of chemicals.....	103
2.9.2	Growth inhibition assay	103
2.9.3	Contact inhibition assay.....	106
2.10	Mucus antibody extraction	
2.10.1	Gill perfusion.....	110
2.10.2	Antibody extraction from mucus	111
2.11	ELISA to detect <i>Paramoeba</i> antibodies in fish	111

Chapter 3. Isolation and verification of the pathogen

3.1	Introduction.....	113
3.2	Methods.....	114
3.2.1	Isolation and characterisation	114
3.2.2	Verification of the pathogen	121
3.2.3	Additional investigations	127
3.3	Results.....	131
3.3.1	Isolation and characterisation	131
3.3.2	Verification of the pathogen	143
3.3.3	Additional investigations	156
3.4	Discussion	163
3.4.1	Isolation and characterisation	163
3.4.2	Verification of the pathogen	168
3.4.3	Gill health	177
3.4.4	Additional investigations.....	181
3.5	Conclusions	184

Chapter 4. Pathogenicity of *Paramoeba* sp. ‘AGD’

4.1	Introduction	186
4.2	Methods	187
4.2.1	Tank systems.....	187
4.2.2	The fish	191
4.2.3	Disease assessment	192
4.2.4	Culture challenges	192
4.2.5	Cohabitation challenges.....	198
4.2.6	Electron microscopy	199
4.3	Results	200
4.3.1	Culture challenges	200
4.3.2	Cohabitation challenges.....	203
4.3.3	Electron microscopy	205
4.4	Discussion	208
4.4.1	Culture challenges	210
4.4.2	Cohabitation challenges.....	219
4.5	Conclusions	221

Chapter 5. Identification and screening of potential amoebicides

5.1	Introduction	223
5.2	Methods	224
5.2.1	The isolate.....	224
5.2.2	Chemicals	224
5.2.3	Chemical screening methods	226
5.3	Results	228
5.3.1	Growth inhibition assay	228
5.3.2	Contact inhibition assay.....	229
5.4	Discussion	233
5.4.1	Growth inhibition assay	237
5.4.2	Contact inhibition assay.....	245
5.5	Conclusions	251

Chapter 6. Non-chemical control of AGD

6.1	Introduction	253
6.2	Methods	254
6.2.1	Improving diagnosis	255
6.2.3	Environmental prevalence	257
6.3	Results	260
6.3.1	Improving diagnosis	260
6.3.2	Environmental prevalence	265
6.4	Discussion	266
6.4.1	Improving diagnosis	267
6.4.2	Environmental prevalence	271
6.5	Conclusions	273

Chapter 7. Detection of anti-*Paramoeba* antibodies in Atlantic salmon

7.1	Introduction	274
7.2	Methods	275
7.2.1	Production of <i>Paramoeba</i> antibodies in salmon	275
7.2.2	Serum and mucus collection from naturally infected fish	276
7.2.3	Development of the ELISA	276
7.3	Results	277
7.3.1	Fish immunisation	277
7.3.2	Mucus collection	277
7.3.3	ELISA	278
7.4	Discussion	281
7.5	Conclusions	285

Final discussion, conclusions and future directions	287
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Appendices	308
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Bibliography	339
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Abstract

Amoebic gill disease (AGD) is the most significant disease of farmed sea-caged salmonids in Tasmania. The research reported here provides the first substantiated evidence for a *Paramoeba* species as the cause of this economically important disease. A total of 680 cultures of amoebae were prepared during an extensive sampling programme of diseased Atlantic salmon, resulting in 61 successfully purified and subcultured amoeba isolates. This library of amoebae comprised the protozoan genera *Platyamoeba*, *Vannella*, *Flabellula*, *Heteroamoeba*, *Vexillifera*, *Acanthamoeba* and *Paramoeba*. Fixed and frozen sections of gills from fish with AGD were immunostained with polyclonal antisera against the predominant genera associated with gills. Only *Paramoeba* was detected in large numbers on gill tissue and always in close association with gill hyperplasia, a characteristic pathognomonic of AGD. Antisera to *Paramoeba* were highly specific and did not cross react with other genera of gill-associated amoebae. Specificity of the antisera has enabled the development of a rapid and highly accurate immunofluorescent antibody test for the identification of clinical cases of AGD in farmed fish and is now the major screening tool of farmed Atlantic salmon in Tasmania.

Despite evidence that *Paramoeba* is the cause of AGD, fulfillment of Koch's postulates could not be achieved when naïve rainbow trout or Atlantic salmon were exposed to freshly isolated cultures of *Paramoeba* with limited laboratory passage. Infection could be established however in cohabitation trials when naïve fish were exposed to fish with AGD.

A sensitive enzyme linked immunosorbent assay was developed using *Paramoeba* isolated from fish with AGD. In a preliminary survey of several Atlantic salmon populations naturally exposed to *Paramoeba*, between 50-100% of fish had circulating serum antibody to *Paramoeba*; no antibody could be detected in gill mucus. The presence of anti-*Paramoeba* antibodies in the serum of fish exposed to *Paramoeba* and/or infected with AGD provided additional evidence for the role of *Paramoeba* in AGD.

AGD is controlled by bathing fish in freshwater. The standard treatment regime for fish was validated by determining the rate of inactivation of *Paramoeba* in freshwater. In addition, 37 potential anti-amoebic compounds were screened for their amoebistatic and amoebicidal activity against *Paramoeba* using assays developed in this study to determine contact and growth inhibition effects. From these *in vitro* trials it was established that *Paramoeba* were totally inactivated by exposure to freshwater within 120 minutes. Hydrogen peroxide at 100ppm inactivated *Paramoeba* within 30 minutes as did exposure to 0.1ppm ozone for four hours. Of the remaining compounds tested, 8-hydroxyquinoline, chloro-iodo-hydroxyquinoline and pyrimethamine at 30µg/ml for four hours' exposure were able to inactivate *Paramoeba* effectively and may have potential as medicated bath treatments for AGD. These trials also identified several chemicals able to inhibit the growth of *Paramoeba* at concentrations of <30µg/ml and may have potential as in-feed treatments for AGD.

Abbreviations

Abbreviations commonly used in the text are listed below:

AGD	Amoebic gill disease
AFC	Analytical flow cytometry
BGD	Bacterial gill disease
BKD	Bacterial kidney disease
CNG	Clubbing and necrosis gill syndrome
DAB	Diaminobenzidine tetrahydrochloride
DAPI	4'6-diamino-2-phenylindole
DB	Dilution buffer
DMSO	Dimethyl sulphoxide
DPIWE	Department of Primary Industries, Water and Environment
EWB	ELISA wash buffer
FWA	Freshwater agar
GIA	Growth inhibition agar
H&E	Haematoxylin and eosin
IF	Immunofluorescence
IFAT	Immunofluorescent antibody test
IP	Immunoperoxidase
MIC	Minimum inhibitory concentration
MPD	Methyl prednisolone
MPN	Most probable number
MYA	Malt yeast agar
NGD	Nodular gill disease
O.D.	Optical density
PBS	Phosphate buffered saline
PMSF	Phenylmethyl sulphonyl fluoride
ppm	Parts per million
ppt	Parts per thousand
R.O.	Reverse osmosis
Saltas	Salmon Enterprises of Tasmania
SSW	Sterile sea-water
SWA	Sea-water agar
TMB	3,3',5,5'-tetramethyl benzidine

Chapter 1. Background and Review of Literature

The aim of this review is to discuss the literature leading up to the start of this study, and to provide a background of the salmonid aquaculture industry and the diseases affecting it, with particular reference to the status of the Tasmanian salmonid industry. Recent relevant publications have also been included, however papers that have been published since the completion of this work and arising from the advances made during this study, will be discussed in detail in the relevant chapters.

1.1 Sea-farming of salmonids

1.1.1 The Salmonidae

The Salmonidae have a natural distribution throughout most of the northern hemisphere, from the equator and northwards to beyond the Arctic Circle. There are no native salmon or trout in the southern hemisphere, but they have been successfully introduced into Africa, South America and Australasia (Sedgwick, 1988). Almost all members of the salmon family can adapt themselves to life in the sea. Most members of this family are anadromous, hatching and growing for a short time in freshwater before their migration to the sea. Here they remain growing and feeding until sexual maturity, returning to their parent rivers when they are ready to spawn. A few species are wholly adapted to freshwater mainly because they have become landlocked and have no access to saline waters. (Brown and Gratzek, 1980; Sedgwick, 1988).

Members of the salmonid family comprise Atlantic salmon (*Salmo salar*); the Pacific salmon, chum (*Oncorhynchus keta*), sockeye (*Oncorhynchus nerka*), pink (*Oncorhynchus gorbusca*), coho (*Oncorhynchus kisutch*) and chinook (*Oncorhynchus tshawytscha*) salmon; trout as, brown (*Salmo trutta*), rainbow (*Oncorhynchus mykiss* formerly *Salmo gairdneri*) and cutthroat (*Salmo clarki*) trout; and finally charr, Arctic (*Salvelinus alpinus*), brook (*Salvelinus fontinalis*) and lake (*Salvelinus namaycush*) charr (Sedgwick, 1988).

1.1.2 Introduction of salmonids to Australia

There are no native members of the family Salmonidae in Australia (AQIS, 1997). There are however, 26 native species of salmoniform fishes (members of the order Salmoniformes, which includes the family Salmonidae), most of which occur in freshwater (AQIS, 1997). There are five introduced species of the family Salmonidae in Australia: rainbow trout, chinook salmon, brown trout, Atlantic salmon and brook 'trout' (charr) (AQIS, 1997).

The European brown trout was successfully established into Tasmanian freshwater systems by the late 1860's, in response to the needs of recreational fisherman who found that indigenous Australian fish offered no substitute to the fishing of salmon and trout enjoyed in England (Dix, 1986). Four attempts were made before successful establishment was achieved (Roughley, 1951). Similar success with this species was obtained in the 1880's in both Victoria and New South Wales. Self sustaining populations of brown trout can now be found in most streams in the Australian Capital Territory (ACT); in Victoria and New South Wales (NSW), mostly in the cool high altitude waters of the states; in Tasmania throughout all major river systems, with the exception of south-west Tasmania; in Queensland, Western Australia (WA) and South Australia (SA) some relict populations still survive, however they are not widespread due to their low thermal tolerance (AQIS, 1997). In NSW, SA, Victoria, and Tasmania brown trout are grown in hatcheries for stocking private and public waters (AQIS, 1997).

Brook trout were introduced into Australia, possibly as early as 1878, however the first recorded importation occurred into Tasmania in 1883, from New Zealand. There is no evidence that self-maintaining populations became established. In 1962 the last importation of brook trout ova occurred from Nova Scotia, Canada. Yearlings from this stock were released in Clarence Lagoon, Tasmania, where a self-maintaining population was established. In 1968, fry grown from brood stock originally from Tasmania were released in NSW, but failed to establish self-maintaining populations. Brook trout thought to be hatchery-released fish, are now found occasionally in the

ACT, in NSW and SA, (mainly as a result of stocking programs), and in Tasmania (as a result of stocking programs and a self-sustaining population in Clarence Lagoon). (AQIS, 1997)

Chinook salmon ova were first imported from the US in 1877 and fry released into Victorian waterways. Importation continued until the 1966 when the final importation was received in Victoria, forming the basis of brood stock cultured in the Snobs Creek Hatchery. Chinook salmon have not established self-maintaining populations, however fry have been released into Victorian and Tasmanian waterways. Currently chinook salmon are stocked in 5 Victorian lakes. (AQIS, 1997)

In 1894 ova from an American steelhead strain of rainbow trout (*Oncorhynchus mykiss*) were brought from New Zealand and introduced into NSW, where the species quickly became established (Roughley, 1951; Dix, 1986). Ova from this species were sent to Tasmania from both NSW and New Zealand in 1898, also becoming quickly established (Dix, 1986). Fry, grown in NSW, were released in Queensland in 1896 but failed to produce viable populations. All Australian rainbow trout originated from New Zealand. Self-maintaining populations of rainbow trout are now found in the ACT, Victoria, NSW and Tasmania. Limited numbers can also be found in parts of Queensland, WA and South Australia. Recreational stocking occurs in Victoria, NSW and South Australia. Commercial culture of rainbow trout occurs in NSW, WA, and in limited numbers in South Australia; Tasmania is the major producer of commercially cultured rainbow trout as both ocean and freshwater varieties. (AQIS, 1997)

Atlantic salmon ova were first imported into Melbourne in 1864, fry from some of the ova being released into the Upper Yarra river in Victoria and into the Plenty river in Tasmania. Self-maintaining populations were not established. Subsequent importations of ova from England, Ireland, the US and New Zealand occurred over the next 70 years, and from Nova Scotia, Canada, in 1963. There are no self-maintaining populations of Atlantic salmon in Australian waters. Atlantic salmon are stocked in the waters of the ACT, NSW and Victoria, and are known to occur in

Tasmanian and South Australian waters. A single grow out facility exists at Ballendean in Queensland, and a small stock remains on a commercial farm in Western Australia, as a result of the unsuccessful introduction in the 1980's. Three grow out facilities occur in South Australia, originating from Tasmanian stock. Sea-cage farming of Atlantic salmon is a major part of the Tasmanian aquaculture industry, accounting for 90% of commercially produced Atlantic salmon in Australia. In December 1996 there were 6 hatcheries licensed to produce salmonids, and 39 licensed marine farms operating in Tasmania (AQIS, 1997).

1.1.3 History of sea-water salmon farming

Historically, wild salmonids have been caught and their eggs stripped, artificially fertilised and incubated in hatcheries since the middle of the nineteenth century. Serious thought regarding the commercial possibilities of breeding and cultivation of this species as a human food, by domesticating them and retaining them in captivity, did not occur until early this century. (Sedgwick, 1988)

The first attempts to culture salmonids in the sea were undertaken by Norway in 1912, using rainbow trout. These initial attempts failed due to the sea breaking open the pens made to enclose the fish. No further attempts of any consequence were made until the mid-1950s. Over the next 10 years production improved until it reached 500 tonnes in 1965 (Sedgwick, 1988). In 1988 there were 690 farms in and along the many Fjords and inlets of the Norwegian coastline, with an annual capacity of 80,000 to 90,000 tonnes, and 562 hatcheries capable of producing 150 million smolts (pre sea-water transfer salmonids), accounting for approximately 80% of Atlantic salmon production in the world (O'Sullivan, 1988).

In the British Isles the first trials of sea-farming salmonids were undertaken in 1960 at Loch Swae on the west coast of Scotland (Sedgwick, 1988). The fish were kept in net cages in brackish water and then transferred to floating cages in the sea-loch. These trials were successful with further commercial development being undertaken during the mid-1960s.

In 1988 the major salmonid sea farming nations were Norway, the United Kingdom, Canada, Chile, the United States of America, Iceland, and the Faroe Islands (O'Sullivan, 1988). In the 10 years from 1987 production of sea-cage farming of Atlantic salmon has increased from an annual production of 68,105 tonnes, worth \$428 million USD, to 555,643 tonnes in 1996, worth approximately 16 billion dollars (FAO Fisheries Circular, No. 815). In 1996 Norway continued to account for 54% of Atlantic salmon production, with a production in excess of 300,000 tonnes of salmon. In 1996 Australia accounted for approximately 1.4% of Atlantic salmon production worldwide. Figure 1 shows Atlantic salmon production by country for the years 1987 to 1996.

Most of the salmon products produced by sea-farming are derived from Atlantic salmon (AQIS, 1997). Although declining in importance the Pacific salmon, such as coho and pink salmon, are successfully farmed in North America, Europe and Asia; rainbow trout and chinook salmon are also popular choices for sea-water farming (Sedgwick, 1988). Arctic charr are now being farmed with some success in Canada. Other salmonid species have either not been extensively investigated for sea-water farming, are not considered of high enough quality, or have attributes that do not suit them to sea-water culture.

1.1.4 Salmonid farming in Australia

In 1993 Tasmania was the only state in Australia where salmon were grown commercially in the sea, although unsuccessful attempts were made in Western Australia in 1988 (Stanley, 1993a). More recently Atlantic salmon production has been recorded in NSW, South Australia and Victoria, although during the 1997/98 financial year Tasmania still accounted for over 99.9% of sea-caged farmed Atlantic salmon in Australia (O' Sullivan and Roberts, 1999). Tasmania is the largest producer of farmed salmonids in Australia, as the production of sea-cage farmed Atlantic salmon exceeds that of all other farmed salmonid species in Australia. Table 1.1 shows the total salmonid production in Tasmania by fish species for the financial year 1997/98; Table 1.2 shows a breakdown of these figures by state. (Tables were compiled from data in O'Sullivan and Roberts, 1999).

Table 1.1: Total Tasmanian salmonid production for the year 1997/98
(O'Sullivan and Roberts, 1999)

Species	Production (tonnes)	Hatchery♣ (,000s)	Value (\$,000)
Atlantic salmon	7,072	0	60,945.2
Chinook salmon	NDA	NDA	NDA
Brook trout	1.3	0	8.7
Brown trout	4	844.4	520.3
Rainbow trout (f/w)	2,736.1	457.2	17,445.8
Rainbow trout (s/w)	500	NA	3,750

Sources: State Fisheries Departments and industry

♣ = hatchery production, not for sale to commercial farms

NDA = no details available

f/w = freshwater s/w = sea-water

Table 1.2: Australian salmonid aquaculture production by state, 1997/98

Species	Production (tonnes)	Hatchery♣ (,000s)	Value (\$,000)
NSW			
Atlantic salmon	0.7	0	4.7
Brook trout	1.3	0	8.7
Rainbow trout (f/w)	283.1	74.2	1,818
South Australia			
Atlantic salmon	2.5*	0	25*
Rainbow trout (f/w)	23	0	2,681
Victoria			
Atlantic salmon ¹	NDA	NDA	NDA
Chinook salmon ¹	NDA	NA	NDA
Brown trout	4*	260.4*	228.3*
Rainbow trout (f/w)	1,800*	143.3*	10,260*
Tasmania			
Atlantic salmon ²	7,068.8	0	60,915.5
Brown trout	0	584	292
Rainbow trout ² (f/w)	600*	239.7	4,919.8
Rainbow trout ² (s/w)	500*	NA	3,750*

Sources: State Fisheries Departments and industry

♣ = hatchery production not for sale to commercial farms

*estimates provided by industry

1: commercial production, confidential data as only one producer

2: government figures provided lumped production for these three groups together, separation based on industry estimates, weight is for head-on, gilled and gutted.

NDA = no details available

NA = not applicable

Tasmania is a small island state, which lies off the south east coast of mainland Australia and has a population of approximately 450,000 people. The climate is temperate, and the waters cool with good water movement, and pollution free. In summer the water temperatures rarely exceed 19°C, whilst in winter they rarely fall below 12°C. Much of the coastline is rugged with many sheltered bays and inlets. All of these features make the state ideal for fish farming.

The Tasmanian salmonid industry had its origins in the establishment of a freshwater trout farm at Bridport in 1964. A second farm was established at Russell Falls in 1974 (Stanley, 1993a). From 1981, experiments were conducted in Tasmania to determine the viability of rearing rainbow trout in net pens in the sea (Dix, 1986). These attempts were successful and the fish marketed as ocean trout. The success of these initial experiments led to the Tasmanian government in 1983/84 identifying sea cage culture of rainbow trout and Atlantic salmon as a promising area for future industry development.

The success of ocean trout led to the first trial imports of Atlantic salmon eggs for aquaculture into the state in 1984 (Stanley, 1993a). A quarantine facility was built in Tasmania by the Department of Fisheries to allow this importation of salmon eggs into Tasmania (Gjovik, 1988). The eggs were imported from the NSW hatchery at Gaden, from stock originally imported from the Phillip River in Nova Scotia, Canada. These fish were originally imported to stock freshwater lakes in the Snowy Mountains (Stanley, 1993a). Further eggs were imported from the same hatchery in 1985 and 1986.

A joint venture between the Tasmanian government and a multinational Norwegian company, Noraqua, was set up in 1984 to ensure the successful development of this industry. In May of 1985 the government passed the Salt-Water Salmonid Culture Bill in Parliament, establishing Salmon Enterprises of Tasmania Pty Ltd (also known as Saltas) as a joint development company for the new industry. Shares were divided between the Tasmanian government (51%), Noraqua (19%) and 16 Tasmanian investors (30%). Noraqua shares were later transferred to the company Tassal, now

the largest salmon grower in Australia. The objectives of Saltas were the production of smolts, selective breeding programmes, development of fish rearing techniques suitable to Tasmanian conditions, and research into nutrition and pathology. The company was given a monopoly on smolt production until 1995 and a hatchery was constructed in the centre of the state. The monopoly allowed a levy to be placed on the sale of smolt to create funds to finance research and development, allowing a mechanism of controlled industry development. (Gjovik, 1988; and Stanley, 1993a)

1.1.5 Economics of the Tasmanian salmonid industry

The first harvest of Atlantic salmon, which yielded 55 tonnes, occurred in the season of 1985/86 (Stanley, 1993a). In 1985 farms produced a small harvest of 5-10 tonnes of ocean trout. By 1989 Atlantic salmon had largely replaced ocean trout on marine sites due to increased mortality, failure to thrive and early maturing of ocean trout in high water temperatures, and the more attractive production performance of Atlantic salmon (Purser, 1992; McKelvie *et al*, 1994). Ocean trout production is now largely confined to the brackish waters of Macquarie Harbour, off the west coast of Tasmania (Purser, 1992).

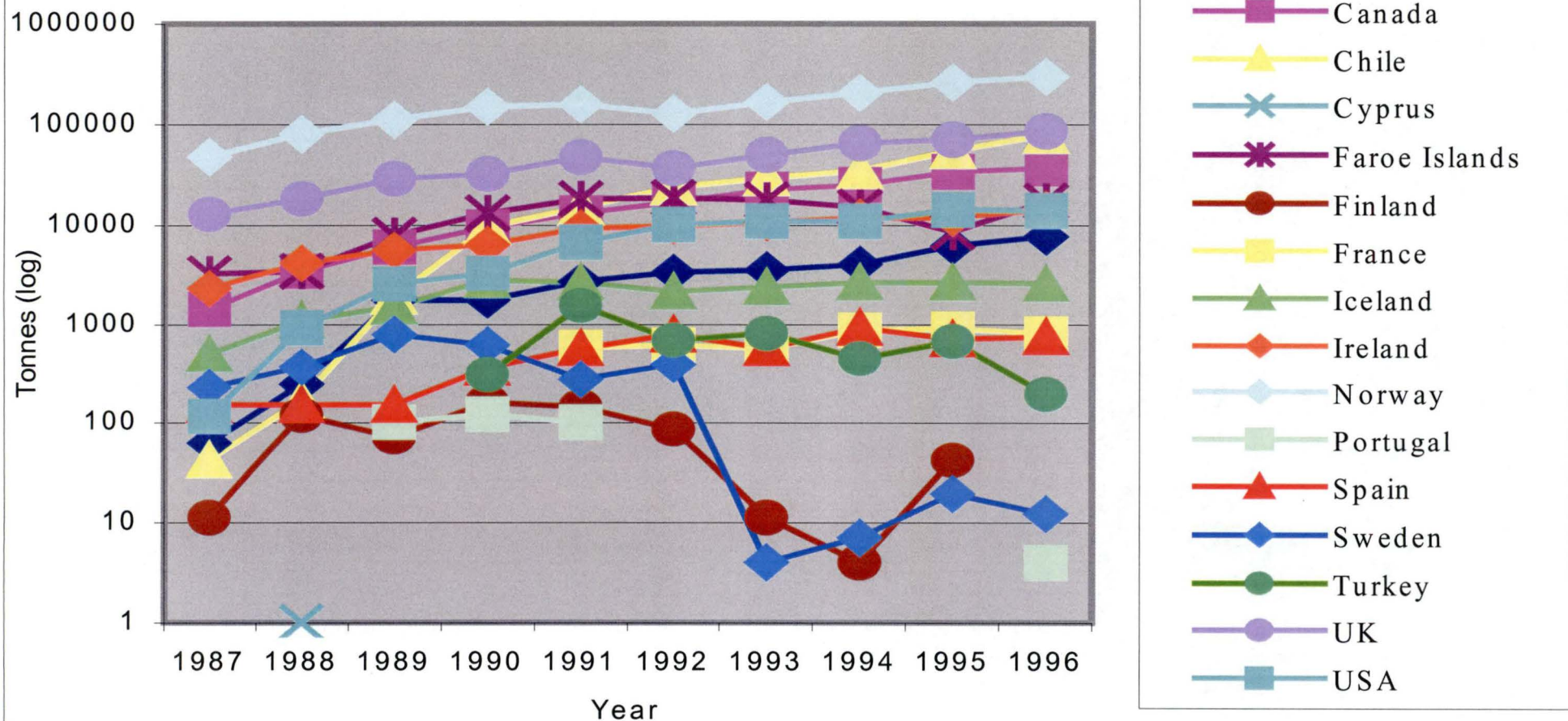
By the season of 1991/92 the industry had expanded, producing 3,020 tonnes of Atlantic salmon with a gross value of \$36 million, and 400 tonnes of ocean trout with a value of \$4 million (Stanley, 1993b). An estimated 70% of this produce was exported, principally to Japan as chilled fish. The industry directly employed at least 300 people on farms and processing plants with a total of 1,000 in affiliated employment. The most recent production figures, for 1996/97, put the total Atlantic salmon production in Tasmania at approximately 7,068 tonnes with a value of approximately \$61 million (AUS\$) (O'Sullivan and Roberts, 1999). In 1996 there were six hatcheries licensed to grow salmonids and 39 licensed marine farms. (AQIS, 1997).

Recent studies on the impact of lifting the importation ban on uncooked salmon products from countries such as Canada, due to the risk of introducing exotic diseases that could devastate the industry, put the value of salmonid aquaculture to Tasmania at \$100 million per annum (Dwyer, 1996). The Australian Bureau of Agriculture and Resource economics placed a value of \$80 million on the Tasmanian Atlantic salmon industry in the event of its loss due to the introduction of two major salmonid diseases, furunculosis and infectious haematopoietic necrosis virus [IHNV] (AQIS, 1997). A total of between 1000 and 2000 jobs are estimated to be at risk, in areas of Tasmania where unemployment is high, if the industry is compromised by disease (Tasmanian Salmonid Growers Association, 1996).

Compared to the other major producers of Atlantic salmon, such as Norway, Scotland and Canada, the Tasmanian salmonid industry is relatively small. The production figures for 1996 of approximately 7,647 tonnes is small when compared to the Norwegian salmon producers total of 300,000, and Chiles production of approximately 77,000 tonnes. Figure 1.1 shows the worldwide production figures for the years 1987-1996 (figures taken from the FAO Fisheries Circular, No. 815). The availability of sites for farms and a glut of salmon on the world markets places limits on the further expansion of the Tasmanian industry, and for this reason the industry has aimed its product at the quality end of the market and by adding value to the product by the production of smoked salmon, pâtés, caviar and gravlax (Stanley, 1993a). A significant market advantage of the Tasmanian salmonid industry is that it is free of many of the diseases affecting other salmonid stock elsewhere, such as furunculosis and bacterial kidney disease; and hence its produce is largely chemical free.

Figure 1.1: Worldwide Atlantic salmon production, 1987 - 1996

(Figures taken from FAO Fisheries Circular No. 815)



1.1.6 Sea-farming methods

Comprehensive details of the methods of sea-farming are contained in the publications of Sedgwick, (1988), Laird and Needham (1988) and Brown and Gratzek (1980); specific details of Tasmanian salmonid sea-farming have been taken from Purser, (1994) and through personal communication with Harry King (Saltas), Sven Oddsson (Saltas), Jim Smith (Tassal Ltd) and the Tassal website (www.tassal.com.au). Based on this information the process of production of sea-cultured Atlantic salmon is as follows.

The production of sea-cultured salmonids is basically a three-step process, the first two steps being undertaken in a dedicated freshwater hatchery:

1. The production, fertilisation and hatching of eggs
2. Rearing of parr and smolt
3. On-growing of the fish in the sea.

Eggs and milt (sperm) are collected from specially selected brood stock fish, most commonly kept in the sea until they reach sexual maturity, late in summer. They can also be kept in freshwater for the life of the fish. In Tasmania brood stock are kept in the sea being transferred to the freshwater hatchery to complete maturation, as they approach sexual maturity. Once mature, the brood fish are stripped for their eggs and milt manually. The eggs and milt are mixed, and the fertilised eggs then being placed into the incubation system of the freshwater hatchery where they are bathed with 10µm filtered (sometimes sterilised) flowing water, heated to between 6-10°C. When the eggs have hatched, they are transferred to specially designed hatching troughs that mimic the dark quiet environment of the gravel nests in the wild. Hatched eggs are referred to as alevin or yolk sac fry. The yolk sac fry do not require food, living off the nutrient rich yolk carried with them. During this time they require a plentiful supply of oxygen and sufficient water flow to maintain adequate oxygenation, this oxygen rich environment allows the fry to develop rapidly and not expend precious energy seeking oxygen.

When the yolk sac fry are ready to feed, indicated by the fry swimming to the surface of the troughs, they are transferred to larger circular tanks for feeding; the fish at this stage are called first feeding fry. The feed is presented in the form of energy and protein rich pellets, primarily made up of fish meal and fish oils, supplemented with vitamins and minerals. The pellets vary in size according to the size of the fish. Faster growing fish are separated from their siblings to allow them to grow, a process referred to as grading. In Tasmania, once the fry reach approximately 4cm in length, they are gradually moved to outdoor production tanks. During this time the fry develop characteristic vertical bands or stripes of colour, at about one gram in size, and are then called parr. From this point on the process of growing the parr is one of basic husbandry, feeding and grading the fish into fast and slow growing groups. Grading is particularly important as it allows smaller fish to continue to grow, provides a means of eradicating runts, and keeps fish of the same size together, allowing farmers to select the best groups for transfer to the sea.

Late in winter, the parr begin to undergo morphological and physiological changes preparing them for the transition to sea-water, a process called smoltification. These changes are triggered in the fish by increasing daylight and rising water temperature. During smoltification an increase in fat metabolism takes place, resulting in a change in appearance. Smolt (fish that have undergone smoltification) are visibly longer and sleeker, their skin turns silvery and fin margins darken. Internal physiological changes also take place during smoltification, the most important of which is the appearance of large chloride secreting cells in the gills and kidneys, which increase the ability of the fish to excrete salt. These cells increase in number and activity, allowing smolt to not only survive but thrive in the sea. Behavioural changes can also be observed during smoltification, fish housed in round holding tanks will change the direction in which they swim, swimming with the current in the tank, rather than against it.

Approximately 15 months after hatching, at around 75-100 grams, the smolt are ready to be placed in the sea. More recently in Tasmania the time from hatching to smolt has been reduced by photo-manipulation, producing out-of-season smolt so that industry can be supplied with smolt throughout the year rather than just in spring, thus increasing their productivity (King, 1994b).

Until recently, smolt were acclimatised into sea-water gradually by increasing the salinity of the water over a period of time. This process has now largely been discarded, smolt now being either placed directly into the sea, or fed with pellets supplemented with high levels of salt for some weeks, before placing them into the sea.

The development of sea farms has progressed considerably since the early experiments conducted by Norwegian salmonid sea farms. Original methods in Norway included enclosing a bay or fjord with a fixed fence of netting, which was largely unsuccessful due to destruction of the nets by heavy wave action. Some farms pumped sea-water into earth or concrete ponds on the shore, but this proved expensive and less profitable. Subsequently Norwegian sea-farmers decided to concentrate on low-cost fixed tidal enclosures, in the form of floating net cages, which have now superseded most other methods. In Tasmania, cages vary in circumference from 50 to 120 metres. Most farm leases are around 10 hectares, and have a perimeter net fence for seal protection either surrounding the farms or the cages themselves. Before the use of seal protection, losses due to seal attacks were a major problem to the Tasmanian salmonid industry.

The feeding rate of salmonids is directly proportional to water temperature hence fish will grow faster at higher temperatures (O' Sullivan, 1988). In sea-water, salmon usually take approximately 2 years to reach 4-5 kg and trout approximately 1-1.5 years to reach 2 kg. Tasmanian water temperatures provide faster growing conditions, from 80g smolt to approximately 3.5 kg in 12-15 months, making them the fastest growing salmon in the world, with all salmon being harvested after their first year in the sea. The relatively high temperatures in Tasmania, compared to other salmonid sea-farming nations, provides an advantage to this industry in terms of growth,

however the stress caused in hot years and the fact that many diseases are more severe in warmer waters poses a significant and real threat to Tasmanian salmon culture.

1.2 Major Diseases of Sea-Farmed Salmonids

The risk of disease in sea-going salmonids is mostly the result of being kept at high densities, in enclosures from which they cannot escape. In such conditions the risk of disease is high and transmission can be rapid. Losses can be dramatic, but more often there is a continual trickle of mortalities. In both cases disease outbreaks are an important economic strain on the industry (Sedgwick, 1988).

Diseases in cultured salmonids may result from a number of conditions including: bacterial or viral infections; external or internal parasites; toxic algal blooms; environmental conditions, such as lack of oxygen, high water temperatures; deficiencies or toxins in the diet; or physical damage following skin abrasion or gill clogging. Frequently adverse environmental conditions place stresses on the fish making them more susceptible to disease. Viruses and bacteria are known to be present in wild fish stocks but rarely cause epizootic outbreaks or large casualties. This also applies to diseases caused by parasites or adverse environmental conditions (Sedgwick, 1988).

There are two main types of fish pathogen: obligate, and facultative pathogens. Obligate pathogens are those that are normally absent from the water in which there are no diseased fish or carriers, these pathogens require the host for reproduction. Most of the common bacterial and viral diseases belong to this group (Sedgwick, 1988). The second group are facultative or free-living organisms, which are naturally present in the water and which may infect fish under certain conditions, such as when they are stressed or due to physical changes in the environment.

1.2.1 Bacterial diseases

There are a number of bacterial diseases of major concern in the culture of salmonids in both freshwater and sea-water. In this review, only those bacterial diseases that cause major losses in the sea-water phase of salmonid farming have been reviewed. Bacterial diseases of freshwater salmonids, and those diseases of minor significance to the industry are extremely numerous and beyond the scope and purpose of this review.

Aeromonas salmonicida

Furunculosis is a septicæmic disease, caused by the obligate pathogen *Aeromonas salmonicida* ssp. *Salmonicida* (Austin and Austin, 1993; Munro and Hastings, 1993). Furunculosis occurs in both the marine and freshwater environments, and can affect fish at any stage in their life cycle (Munro and Hastings, 1993). Furunculosis is the most common and most serious disease affecting sea-cultured salmonids, causing major economic losses (Ellis, 1997). During the 1980's mortalities in Atlantic salmon farms due to furunculosis were between 15-20% of sea-water grower stocks per year (Ellis, 1997). It is endemic in wild as well as farmed fish in most countries, with the exception of New Zealand and Australia (Carson, 1990; AQIS, 1997). Although *A. salmonicida* ssp. *salmonicida* is exotic to Australia, atypical forms of *A. salmonicida* have been isolated from infected goldfish from the states of Victoria and New South Wales in mainland Australia, where the disease is considered enzootic (Carson, 1990). In 1993 an atypical form of *A. salmonicida* was also isolated from greenback flounder in Tasmania (J. Carson, Pers. Comm.).

Clinical and gross pathology depend on whether the infection occurs in the acute or chronic form (Munro and Hastings, 1993). The acute form is most common in growing or adult fish. Acutely affected fish may show few clinical signs of disease with most of the effects being internal manifesting as general septicæmia; if present clinical signs include anorexia, darkening of the skin, lethargy, and small haemorrhages at the base of the fins (Austin and Austin, 1993; Munro and Hastings, 1993). The acute form of the disease is sudden and rapid, with the fish dying within 2-3 days, and overall mortalities can be high. In its chronic form furunculosis is

characterised by the presence of swellings or ‘furuncles’, resembling boils, which occur all over the fish’s body (Austin and Austin, 1993). The furuncles contain a reddish fluid, which may burst, releasing a mass of bacteria into the water so spreading infection. Internally, liver haemorrhages, swelling of the spleen and necrosis of the kidney may occur. The chronic form of the disease usually occurs in older or larger fish, causing low levels of mortality. In the survivor scar tissue forms in the vicinity of the furuncles, lowering the market value of the fish. The disease spreads throughout the water, or by direct contact between fish (Sedgewick, 1988); however vertical transmission via infected ova has not been demonstrated (Kent, 1992). Fish infected in freshwater can carry the infection into the sea (Kent, 1992). Survivors of outbreaks can become carriers (Munro and Hasting, 1993). Outbreaks of the disease are most likely when the water temperatures are 15°C or above (Sedgewick, 1988).

Control can be achieved by testing of stock before entry to the farm and eliminating infected fish or eggs, maintaining good water quality, and by the disinfection of fish farm equipment (Sedgewick, 1988; Austin and Austin, 1993). Outbreaks have successfully been treated with the use of antibiotics such as, sulphamerazine, chloramphenicol, amoxycillin, flumequin, oxytetracycline and oxolinic acid, incorporated into the feed, or as baths or dips (Sedgwick, 1988; Carson, 1990; Austin and Austin, 1993). However, resistance to licensed antibiotics, with the exception of amoxycillin, is now widespread, and multiple resistance is also common (Munro and Hastings, 1993). In recent years the use of vaccines comprising *A. salmonicida* bacterins emulsified in oil adjuvants and delivered by intraperitoneal injection have been shown to provide long lasting protection against the disease. Despite some side effects, by the mid-late 1990’s these vaccines had been adopted by most Atlantic salmon farmers to control furunculosis (Ellis, 1997), however there is no vaccine that can provide complete protection (Ackerman *et al*, 2000).

Renibacterium salmoninarum

Another serious disease is bacterial kidney disease (BKD), a slowly progressive and frequently fatal infection of cultured and wild salmonids in freshwater and sea-water, caused by the bacterium *Renibacterium salmoninarum* (Fryer and Sanders, 1981; Austin and Austin, 1993; Evelyn, 1993). It causes major economic losses to cultured salmonids in Europe, North and South America, and Japan, but has not been reported in Australia (Austin and Rayment, 1985; Fryer and Lannan, 1993; AQIS, 1997; Kaattari and Piganelli, 1997). Bacterial kidney disease usually infects parr in freshwater, ultimately leading to kidney damage, and significant losses of smolt when they are transferred to the sea (Sedgewick, 1988). Kent (1992) cites BKD as the most significant cause of mortality in pen-reared chinook and coho salmon, and to a lesser degree Atlantic salmon.

Gross signs of BKD include lethargy, darkened skin colour, swollen abdomen, and sometimes exophthalmus, eye lesions and multiple blood filled lesions in the skin (Kent, 1992). The kidney is the primary site of infection, although the skin, eyes, liver, heart, and spleen are also sites of pathogen concentration (Austin and Austin, 1993). Lesions contain a liquid mass of leucocytes, bacteria and cellular debris, and the abdomen is often swollen with ascites fluid. The bacterium is most likely transmitted vertically through infected gametes, and horizontally by close contact with infected fish (Austin and Austin, 1993; Fryer and Lannan, 1993). *R. salmoninarum* is only associated with asymptomatic and clinically affected fish, there is no evidence that it is a component of the normal aquatic flora (Evelyn, 1993). Fish can carry the infection from freshwater to the sea, showing signs of disease shortly after introduction to the sea. It has been postulated that the bacterium is part of the normal flora of the fish being activated during times of stress. (Austin and Austin, 1993)

Most mortality occurs when water temperatures are high, with continual losses of small numbers of fish at lower temperatures (Austin and Austin, 1993). The disease is difficult to treat and the development of effective vaccines has yet to be achieved (Kaattari and Piganelli, 1997). However some measure of control has been achieved by treatment with sulpha based drugs, erythromycin and penicillin incorporated into the feed (Sedgwick, 1988; Carson, 1990; Austin and Austin, 1993; Evelyn, 1993;

Fryer and Lannan, 1993). The intracellular nature of the pathogen is the major impediment to chemical control, and although chemicals may reduce mortality the pathogen cannot be entirely eliminated from the host (Austin and Austin, 1993; Fryer and Lannan, 1993). The role of diet and the breeding of genetically resistant fish is being investigated (Austin and Austin, 1993).

Vibrio anguillarum* and *V. ordalii

Vibrio anguillarum, and less commonly by *V. ordalii*, are the causative agents of vibriosis, a serious systemic disease of wild and cultured marine and brackish water fishes (Egidius, 1987; Kent, 1992; Austin and Austin, 1993). There are many serotypes and biotypes described and a wide range of susceptible fish species (Frerichs, 1989). Vibriosis is present world wide, in marine and estuarine environments, and is a major cause of infection in sea-cultured salmonids (Sedgewick, 1988; Austin and Austin, 1993). Mortalities as high as 90% have been reported in unvaccinated smolts (Kent, 1992). Both *V. anguillarum* and *V. ordalii* have been reported in Australia, however only serotype O1 of *V. anguillarum* has been associated with disease in Australian salmonid fish (Carson, 1990).

Early infection is characterised by loss of appetite and lethargy (Carson, 1990; Hjeltne and Roberts, 1993). As the disease progresses, haemorrhagic areas appear on the skin with reddening at the base of the fins, vent and sometimes the mouth. Bleeding occurs in the gills and internally there may be peritonitis, splenomegaly and lesions in the abdominal cavity. A hallmark of vibriosis is the presence of large, multiple haemorrhages in the liver, which appear unique to affected fish (Kent, 1992). The bacterium usually gains access to the fish via skin lesions, which can be caused by the fish rubbing against the sides of the cages, or by the loss of scales during grading or transportation (Sedgwick, 1988). In the acute form of vibriosis sudden losses can occur with little evidence of disease (Carson, 1990). Most outbreaks occur in smolts during the first summer in the sea, at water temperatures between 15-20°C (Kent, 1992). Risk factors for vibriosis are poor hygiene, overcrowding, high water temperatures and a decrease in the amount of dissolved oxygen in the water; heavy metal contamination of the water may also exacerbate the disease (Austin and Austin, 1993). Control is largely achieved by the use of vaccines administered to the fish prior

to sea transfer and, combined with normal farming practices, these vaccines provide a high level of protection against vibriosis (Austin and Austin, 1993). In Tasmania a locally produced vaccine ‘Anguillvac-c’, made from *V. anguillarum* serotype O1, protects salmonids from outbreaks of this disease in sea-water (Munday *et al*, 1992). Outbreaks can also be treated with the use of antibiotics, such as sulphamerazine, oxytetracycline and oxolinic acid, provided the fish are still feeding (Sedgewick, 1988).

Vibrio salmonicida

‘Coldwater’ vibriosis or ‘Hitra’ disease, caused by *Vibrio salmonicida*, is a relatively new disease of marine farmed salmon, first appearing in Norway in 1979 (Austin and Austin, 1993). The disease is now widespread throughout Norway, and has been reported from Scotland, the Shetland Islands, Canada and on the Atlantic coast of North America (Egidius *et al*, 1981; Bruno *et al*, 1985; O’ Halloran *et al*, 1992; Austin and Austin, 1993). The disease has not been reported in Tasmania (AQIS, 1997). It is only observed in fish in sea-water, with outbreaks usually occurring during late autumn to early spring. (Austin and Austin, 1993). ‘Coldwater’ vibriosis is an economically important disease of marine farmed Atlantic salmon, with mortality rates of up to 63% being reported in North America, and 95% in Europe (Hastein, 1993; AQIS, 1997).

The disease resembles a generalised haemorrhagic septicaemia, with affected fish showing haemorrhaging around the abdomen. The organism causes internal haemorrhaging and is found in the heart, muscle, intestine, liver, kidney, spleen, and in the blood (Austin and Austin, 1993; Hjeltne and Roberts, 1993). Transmission is thought to be via water, with the source of infection being either carrier fish and/or sediment (Hjeltne *et al*, 1987; Enger *et al*, 1989). Oxolinic acid has been used to control mortality, and some success has been achieved with vaccination (Austin and Austin, 1993; Hjeltne and Roberts, 1993; Steine *et al*, 2001).

Piscirickettsia salmonis

Piscirickettsiosis is another newly recognised systemic disease of farmed marine trout and salmon, caused by *Piscirickettsia salmonis* (Fryer *et al*, 1990, 1992). It was first recognised in Chile, where mortalities of up to 90% were observed (Bravo and Campos, 1989). However similar forms have been observed in Ireland, Norway and Canada, although with much lower mortality rates (Brocklebank *et al*, 1992; Austin and Austin, 1993; Rodger and Drinan, 1993; Olsen *et al*, 1997). The disease has been recorded in Atlantic salmon, rainbow trout and chinook. Recently the difference in mortality rates has been attributed to differences in the virulence of *P. salmonis* isolates (House *et al*, 1999). Since its discovery in 1989 piscirickettsiosis has killed millions of farmed fish each year in southern Chile (Smith *et al*, 1999). Piscirickettsiosis has not been reported in Australia (AQIS, 1997).

External signs of the disease include melanosis, epidermal indurations, and pale gills, all indicating anaemia (Austin and Austin, 1993; Rodger and Drinan, 1993).

Respiratory distress and lethargy are also apparent in diseased fish (Felipe *et al*, 1997). Internally, haemorrhaging is evident on the walls of the abdomen, on the air bladder and in the visceral fat. Yellowish subcapsular nodules are observed throughout the liver (a hallmark of the disease); the kidney and liver is also enlarged in severely infected fish. The disease is not observed in freshwater, with mortalities only occurring 6-12 weeks after transfer to sea-water (Fryer *et al*, 1992). The organism is thought to be passed from fish to fish, or via invertebrate vectors or infected wild fish; with high water temperatures thought to trigger high mortality (Kent, 1992; Austin and Austin, 1993; Rodger and Drinan, 1993). Vertical transmission has been reported in coho salmon (Almendras and Fuentealba, 1997). The gills and the skin are thought to be the main sites of entry for *P. salmonis* (Smith *et al*, 1999). The infection responds to a range of antibiotics, including chloramphenicol, erythromycin and oxytetracycline (Austin and Austin, 1993); with flumequine and oxolinic acid being the most popular antibiotics used (Almendras and Fuentealba, 1997).

1.2.2 Viral diseases

Diseases in fish caused by viruses are of special concern to the salmonid industry as they seldom, if ever, respond to treatment. The viral diseases selected here for review have been identified as major disease risks to the Australian salmonid farming industry and wild salmonids and salmoniforms, in the recent report entitled “Salmon Import Risk Analysis” (AQIS, 1997). The AQIS report identified eight viral diseases of concern, all of which are exotic to Australia. Four of the most economically significant to sea-cultured salmonids are reviewed below. There are no known treatments or commercial vaccines for any of the viruses reviewed.

Infectious haematopoietic necrosis virus

Infectious haematopoietic necrosis (IHN) is one of the most important viral diseases of salmon and trout. It is an acute systemic disease caused by a rhabdovirus named infectious haematopoietic necrosis virus (IHNV) (Wolf, 1988; Smail and Munro, 1989). The virus is endemic to North America and Japan where it occurs in many populations of Pacific salmon, and to a lesser extent rainbow trout, coho and Atlantic salmon. Epizootic outbreaks usually occur in juvenile salmonids in freshwater hatcheries; mortality rates can reach 100% (Amend and Nelson, 1977; Wolf, 1988). The disease has also been observed in Atlantic salmon post smolts reared in seawater in British Columbia, with mortalities ranging from 20% to 80% being reported (Armstrong *et al*, 1993).

The main signs of IHN are darkening of the skin, pale gills and haemorrhaging at the base of the fins, and sometimes associated with ascities (Wolf, 1988; Munday, 1990). In acutely ill fish the virus can be isolated from all major organs, and in the eggs and sperm (Wolf, 1988). The virus is shed into the water via faeces, urine, sexual fluid and external mucus, or transmitted by eating infected fish tissue (Sedgewick, 1988). Transmission between smolts in seawater has also been described (Traxler *et al*, 1993). The gills have been implicated as primary uptake sites (Mulcahy *et al*, 1983a, 1983b), but infection via the skin or oral route may also occur (Amend, 1975;

Yamamoto *et al*, 1990, 1992). Adult fish can become asymptomatic carriers (Wolf, 1988). Control can only be achieved by strict testing of biological material entering the hatchery, and destroying infected batches (Munday, 1990; Kent, 1992); and avoiding the transfer of infected fish to the sea (Traxler *et al*, 1993). Naked DNA vaccination has been shown to be effective in protecting immunised Atlantic salmon against experimental challenges with IHNV (Traxler *et al*, 1999).

Infectious pancreatic necrosis virus

Infectious pancreatic necrosis virus (IPNV) is another systemic pathogen, occurring in nearly all countries culturing salmonids (Munday, 1990). IPNV is a member of the birnaviridae family, which also contains members of IPNV related viruses isolated from a broad range of fish species, molluscs and crustaceans (Wolf, 1988; Smail and Munro, 1989; Kent, 1992). Infectious pancreatic necrosis (IPN) has never been observed in fish cultured in Australia or New Zealand (Munday, 1990); however, recently an aquatic birnavirus related to IPNV was isolated from farmed and wild fish species in Tasmania, not associated with gross clinical disease (Crane *et al*, 2000). Infectious pancreatic necrosis is predominantly a disease of juvenile salmonids in freshwater, with an increasing number of outbreaks being reported in Atlantic salmon post-smolts in the sea (Sedgewick, 1988; Jarpe *et al*, 1994; Smail *et al*, 1995). Mortality in freshwater fish can exceed 90% (Kent, 1992). In Norway the mortality of post-smolts during the first 3 months after sea transfer has been found to vary from a few percent, to up to 80% (Jarpe *et al*, 1994). In Norway the total loss of Atlantic salmon smolt due to IPN in 1995 was estimated to be worth 60 million USD (Christie, 1997).

Clinical disease is most prevalent at temperatures between 10 and 15°C. Clinical signs of the disease include darkening of the body, whirling movements, abdominal distension, exophthalmus and whitish casts from the vent (Wolf, 1988; Kent, 1992). The IPN virus causes necrosis of the exocrine pancreas (Kent, 1992). The virus can be found in many tissues and body products, including the kidney, spleen, pancreas/pyloric caeca and liver (Wolf, 1988). Survivors become carriers for life (Wolf, 1988). The virus is transmitted both vertically and horizontally (Smail and Munro, 1989), however it is not known if the disease can be transmitted in sea-water (Sedgwick, 1988). There is no known treatment for IPN, hence the only method of control is to avoid the use of contaminated broodstock, and rearing progeny in virus-free water (Kent, 1992). A variety of vaccines have been trialed, with limited success; there are no commercial vaccines currently available (Christie, 1997).

Salmon leukaemia virus

Plasmacytoid leukaemia, or marine anaemia, is thought to be caused by a retrovirus, named salmon leukaemia virus (SLV) (Kent *et al*, 1990; Eaton and Kent, 1992; Eaton *et al*, 1993; Kent and Dawe, 1993). It has recently described as the cause of high mortalities, up to 80%, in farmed chinook salmon in British Columbia, where the disease is endemic in both sea-water and freshwater (Newbound and Kent, 1991). It has also been observed in wild marine caught salmon and salmon returning to spawn (Kent *et al*, 1993; Eaton *et al*, 1994). The disease is characterised by the proliferation and infiltration of plasmoblasts into the visceral organs and retrobulbar tissue of infected fish (Kent *et al*, 1990). Infected fish are dark, lethargic and often swim near the surface of the water; it also causes anaemia, exophthalmia (“pop eye”), renal and spleen enlargement, petechial haemorrhages and ascities (Kent, 1992). Clinical manifestations of the disease may not be present in infected fish. Plasmacytoid leukaemia is often associated with *Enterocytozoon salmonis* infection, and has also been observed following epizootics of bacterial kidney disease (Kent, 1992). There is little information on the epidemiology and pathogenicity of this agent, although transmission has been demonstrated in cohabitation experiments in freshwater from infected chinook salmon to naïve salmon (Kent *et al*, 1993). It is not known if the

disease can be transmitted in sea-water, however vertical transmission has been suggested (Kent, 1992). There are no reported treatments for this disease, however it has been suggested that farms avoid the use of infected brood stock, and avoid the transfer of infected fish between farms (Kent, 1992). Fumagillin DCH has been used to reduce infections by *E. salmonis* and has been reported to reduce the concurrent leukaemia-like condition (Hedrick *et al*, 1991).

Salmon pancreas disease virus

Salmon pancreas disease (SPD) was first recognized in Scotland in 1976 (McVicar, 1987), and has subsequently been reported in Ireland, Norway, the USA, France and Spain (Nelson *et al*, 1995). A toga-like virus, salmon pancreas diseases virus (SPDV) has been shown to be the aetiological agent of SPD (Nelson *et al*, 1995), although recent biochemical characteristics suggest it is an alphavirus (Welsh *et al*, 2000). The disease only occurs in marine farmed Atlantic salmon, causing major economic losses to the industry (Houghton, 1994). In Ireland up to 50% mortality rates in first year smolts have been attributed to SPD (Nelson *et al*, 1995). Affected fish show a rapid decrease in feeding response, eventually losing weight, become darker in colour and may lose their ability to remain vertical (McVicar, 1986). The disease results in the total necrosis of the acinar cells of the exocrine pancreas; necrotic lesions have also been reported from the heart and skeletal muscle (Ferguson *et al*, 1986; Nelson *et al*, 1995). Although clinical disease has only be observed in fish in saltwater, experimental transmission has been successful in both salt and freshwater via cohabitation, injection with infective kidney material and water (McVicar, 1987, 1990; Raynard and Houghton, 1993; Nelson *et al*, 1995). There is no known treatment for SPD, however significant protection has been demonstrated in experimentally infected parr and post smolts after vaccination with infective kidney homogenate (Houghton, 1994).

1.2.3 Diseases caused by protozoans

There are a number of protozoan parasites of salmonids, most of which affect salmonids reared in freshwater. These infections can usually be controlled by good husbandry practices, and in some cases by the treatment of fish with medicated bath treatments or orally administered chemotherapeutants (Sedgwick, 1988). There are however, a number of protozoan parasites that can cause disease in sea-water, and in some cases cause severe levels of mortality. Kent and Margolis (1995) recently compiled a comprehensive review of the significant parasitic protozoa affecting sea-water reared salmonids. These pathogens affecting sea-water reared salmonids and the associated diseases are outlined below. With the exception of amoebic gill disease (AGD), caused by the amoeba *Paramoeba* sp., and myxosporean *Kudoa* spp., most of the diseases described are not present in Australia.

Ciliates

The ciliates, *Trichodina* spp., are the only ciliates recognised to cause disease in sea-water reared salmonids (Kent and Margolis, 1995). Trichodinids are well-recognised skin and gill parasites of freshwater and marine reared salmonids (Brown and Gratzek, 1980; Langdon, 1990). While harmless in small numbers they can proliferate rapidly causing respiratory distress, especially in association with a drop in temperature and poor water quality (Brown and Gratzek, 1980; Langdon, 1990). However, a study by Rintamaki and Valtonen (1997) suggests that trichodinid infections are not clearly affected by water temperatures. Clinical signs of disease include respiratory distress associated with whitish patches on the skin and fins of the fish and an overall increase in mucus (Brown and Gratzek, 1980; Langdon, 1990). When heavily infected, the gills exhibit epithelial hyperplasia and excess mucus, obstructing gas exchange (Langdon, 1990). Gill lesions and mortality associated with heavy infections have been observed rainbow trout and Atlantic salmon reared in sea in net pens in Ireland (McArdle, 1984; Kent and Margolis, 1995). Treatment with formalin and/or malachite green has been reported to be effective (McArdle, 1984), along with fresh flowing water (Brown and Gratzek, 1980).

Flagellates

The flagellate *Hexamita salmonis* is another freshwater protozoan parasite of salmonids, causing severe intestinal infections in freshwater salmonid hatcheries (Kent, 1992). Most infections are not associated with disease, but some reports have attributed anorexia, emaciation, poor growth and mortality in salmon fry to the infection (Brown and Gratzek, 1980; Kent, 1992). A similar disease in sea-water reared salmonids has been observed in chinook salmon in British Columbia, and Atlantic salmon in Norway (Mo *et al*, 1990; Kent and Margolis, 1995). In British Columbia the disease caused close to 50% mortality some months after the salmon had been introduced to the sea (Kent, 1992). The disease was confined to the circulatory system, and the organism morphologically indistinguishable from the relatively non-pathogenic *H. salmonis* that infects freshwater salmonids (Kent, 1992). Kent readily demonstrated cross infection in both fresh and sea-water in chinook exposed to infected blood and by cohabitation with infected fish. Clinical signs include swollen abdomen, pale gills, and enlarged liver with petechial haemorrhaging. In Atlantic salmon the infection is a little different in that the parasite causes large focal lesions in the musculature and other organs (Kent and Margolis, 1995). High stocking density in fry is thought to increase transmission of the organism in salmon fry in freshwater (Uldal and Buchmann, 1996). Horizontal transmission has been demonstrated in chinook, in both freshwater and sea-water, by water-borne exposure to infected blood and viscera, and by cohabitation with infected fish (Kent, 1992). Metronidazole has been used to treat disease in freshwater aquarium fish (Brown and Gratzek, 1980; Langdon, 1990), although several drugs have been recommended for use in the sea, so far none have been used to treat sea-water infections (Kent and Margolis, 1995; Tojo and Santamarina, 1998c).

The flagellate *Ichthyobodo necator* (= *Costia necatrix*) is another common gill and skin parasite of freshwater reared salmonids (Bruno, 1992b; Kent and Margolis, 1995; Kent, 1992). It has been shown to survive and proliferate on fish transferred to the sea, and cause problems in sea-water reared salmonids. *Ichthyobodo* infections have

been reported in sea-water reared Atlantic salmon in Europe, and have been associated with gill damage in pen-reared chinook in British Columbia and Scotland (Bruno, 1992b; Kent, 1992). In Norway a mortality rate of up to 40% was observed in Atlantic salmon smolts in summer and autumn due to *I. necator* (Urawa *et al*, 1998). Although infection is thought to be acquired in freshwater, the pathogen has been observed in strictly marine fishes, such as haddock and flatfishes (Bruno, 1992b; Kent, 1992). Bruno (1992b) found that the trophozoites found on the gills of sea-water salmon was morphologically distinct from the *I. necator* found in salmonids reared in freshwater, suggesting the existence of a marine species of *Ichthyobodo*. Pathological changes are usually confined to the gills, with histological sections showing diffuse, epithelial hyperplasia of the gill epithelium associated with attached parasites. Parasites can also be observed in wet mounts of skin (Bruno, 1992b; Kent, 1992). Clinically, heavily infected fish appear emaciated, anorexic, with a tendency to swim near the surface of the water (Kent, 1998). Urawa *et al* (1998) suggest the best method to control infection is to eradicate the parasite prior to transfer to the sea. The most effective treatments being a formalin or malachite green bath (Kent, 1992). However, use of formalin is not recommended in sea-water, as it has been shown to cause heavy mortality (Urawa *et al*, 1998). Metronidazole and secnidazole have been shown to be effective in treating infected rainbow trout (Tojo and Santmarina, 1998b).

Rosette Agent

A serious systemic disease of emerging importance, caused by a parasite termed the rosette agent, has been observed in chinook salmon reared in the sea in Washington State (Elston *et al*, 1986; Harrell *et al*, 1986). A very similar organism is thought to have caused a similar disease in pen-reared Atlantic salmon in Canada (Cawthorn *et al*, 1991). Chinook salmon, Atlantic salmon, rainbow and brown trout are all susceptible to this parasite (Harrell *et al*, 1986; Hedrick *et al*, 1989; Nash *et al*, 1989). The disease is caused by an as yet unclassified intracellular eukaryotic parasite, observed as clusters or 'rosettes' in macrophages, especially in the kidney and spleen (Kent and Margolis, 1995). The taxonomic classification of this organism has been

controversial, being classified with the algae and fungi by some researchers (Harrell *et al.* 1986; Kent, 1992), or as a protozoan in the phylum Apicomplexa (Cawthorn *et al.*, 1991). However, phylogenetic analyses of its ribosomal subunit RNA suggest it is closely related to the choanoflagellates (Phylum Choanozoa), which are usually free living flagellates (Kerk *et al.*, 1995). Infected fish are anaemic and often dark, leading the disease to be termed 'black smolt syndrome'; the spleen and kidney are also enlarged (Kent and Margolis, 1995). The agent has been associated with mortalities of up to 90%, with the disease occurring in the summer and autumn (Kent, 1992). There is no known treatment for this disease.

Myxosporeans

A number of myxosporean parasites have been reported as the cause of disease in sea-water reared salmonids. *Parvicapsula* sp. infects the kidney of sea-reared salmon, and has been reported to be associated with severe disease in coho salmon in Washington State, USA (Kent and Margolis, 1995). Mortalities are observed in caged smolts, soon after they are placed in sea-water (Kent, 1992). The organism has been found in wild coho, sockeye, chinook, masou and Atlantic salmon as well as in cutthroat trout in North America (Schiewe *et al.*, 1988; Kent, 1992). It has also been found in Pacific cod collected near the pens of infected coho salmon, and it has been suggested that these fish may act as a reservoir of infection (Kent, 1992). The life cycle takes place in the marine environment. Mortalities are usually low, and are influenced by secondary infections with *Renibacterium* and *Vibrio* spp, hence the role of the parasite in the host is unclear (Kent, 1992). The prevalence of the infection increases with an increase in water temperature. Heavily infected fish are dark and lethargic, have swollen kidneys, cease feeding and show behavioural abnormalities (Kent and Margolis, 1995). The parasite is readily identified in stained kidney sections. There are no commercially available drugs to treat disease caused by *Parvicapsula* sp., but fumagillin DCH has been suggested as a possible chemotherapeutant as it has been found to be efficacious in treating other infections caused by myxosporeans (Kent and Margolis, 1995).

Myxosporeans of the genus *Kudoa* infect the musculature of many marine fishes, causing unsightly white cysts and soft texture in fillets after processing. This flesh softening is due to a proteolytic enzyme produced by the *Kudoa*, which remains active at temperatures below 70°C, allowing it to survive the smoking process, normally conducted at 50°C (Kent, 1992). This soft flesh disease associated with *Kudoa* spp. has been reported in sea-farmed Atlantic salmon in the Pacific Northwest, and Ireland, and in sea-farmed brown trout in France (Kent and Margolis, 1995). The prevalence of infection in pen-reared Atlantic salmon has been reported at between 2-25% (Kent, 1992). Fish become infected in the sea, the majority of post-smolts contracting the infection within a few months post transfer to the sea (Moran and Kent, 1999). *Kudoa thyrssites* has been reported in more than 27 species of marine fish, including salmonid and non-salmonid fishes (Whitaker *et al*, 1994; Moran and Kent, 1999). In Australia it has been reported in a number of marine finfish, including pilchards collected from Western Australia, which showed an average prevalence rate of 67% (Willis, 1949; Langdon, 1991; Langdon *et al*, 1992). Flesh softening due to *Kudoa* spp. is of particular concern to the salmonid farming industry because infection can lower the value of infected fish, although the parasite is seldom associated with morbidity or mortality (Kent, 1992). There is no treatment available.

Microsporidians

Microsporidians are common parasites of marine fishes, with two species causing disease in sea-farmed salmonids. *Loma salmonae* infects the gills and other tissues of salmon reared in freshwater. Infections can persist after the fish are transferred to the sea, with mortality rates of 10-12% being reported in smolts recently transferred to the sea (Hauck, 1984). Severe gill infections have been reported in rainbow and steelhead trout, as well as kokanee salmon, while high levels of mortality have also been observed in chinook salmon due to systemic infection (Kent, 1992). There are no reports of this disease occurring in Atlantic salmon (AQIS, 1997). The gills are the primary site of infection, but the parasite can also occur in the heart, kidney, spleen and pseudobranchs (Kent, 1992). Infected fish usually show signs of respiratory distress, white cysts and or lumps may be present on the gills of heavily infected fish

(Kent and Margolis, 1995). Transmission of the parasite has been demonstrated in freshwater by cohabitation of naïve fish with infected fish, intraperitoneal, intramuscular and intravascular injection (Shaw *et al*, 1998). The parasite is transmissible from fish to fish in the sea, as demonstrated by the infection of naïve chinook salmon when exposed to macerated gill tissue from infected chinook (Kent *et al*, 1995; Kent and Margolis, 1995). There are no commercially available drugs to treat this disease, however oral treatment with fumagillin DCH has been suggested as a possible chemotherapeutant (Kent, 1992). As infections originate in freshwater, control is best achieved by avoiding the placement of infected fish into the sea (Kent, 1992).

Enterocytozoon salmonis is thought to be an important co-factor in the neoplastic disease known as plasmacytoid leukaemia caused by the salmon leukaemia virus (Kent, 1992; Kent and Margolis, 1995). The parasite has been reported in sea-reared chinook salmon in Washington State, associated with anaemia, and also in British Columbia and Canada (Kent and Margolis, 1995). The disease has also been reported in sea-reared Atlantic salmon in Chile, where a mortality rate of 64% was observed soon after the smolts were transferred to seawater (Bravo, 1996). Infected fish show severe anaemia with prominent pale gills, lethargy, exophthalmos ('pop-eye'), kidney enlargement and spleen and intestinal swelling (Morrison *et al*, 1990; Bravo, 1996). Secondary infections, or stress factors are thought to be involved in the expression of clinical disease. Lateral transmission of *E. salmonis* has been demonstrated in freshwater by cohabitation of infected chinook salmon with naïve chinook, and also by feeding naïve chinook with the kidneys and spleens of infected chinook (Baxa-Antonio *et al*, 1992). There are no commercially available drugs for treating *E. salmonis*, although fumagillin has been shown to control infection in experimentally infected chinook salmon (Kent, 1992).

Amoebae

The only serious amoeba infection of sea-reared salmonids is caused by *Paramoeba pemaquidensis*. This disease has been associated with severe gill disease of coho salmon in Washington State (Kent *et al*, 1988). A similar, possibly identical amoeba, identified as an unspeciased *Paramoeba* sp. has caused a similar disease in sea-reared Atlantic salmon and rainbow trout in Tasmania, causing devastating losses to this industry when it first occurred (Roubal *et al*, 1989; Munday *et al*, 1990). The intensity and prevalence varies from year to year, with most infections occurring during summer and autumn in Washington State and from spring to autumn in Tasmania. In contrast to Washington State, this disease occurs in Tasmania throughout the fish's first year in the sea and is the most significant disease facing this industry at this time (H. King, Pers. Comm.). The only method of control at this time is to bathe the fish for short periods in freshwater (Roubal *et al*, 1989; Munday *et al*, 1990). This gill disease, termed amoebic gill disease (AGD), has been reported in chinook salmon farmed in New Zealand (C. Anderson, Pers. Comm.); in sea-cage farmed Atlantic salmon in Ireland (Rodger and McArdle, 1996; Palmer *et al*, 1997) and France (Findlay *et al*, 1995) although in contrast to Tasmania, outbreaks in these places appear to be sporadic.

1.2.4 Other diseases

Diseases caused by parasitic flukes and worms, such as *Dactylogyrus* spp. and *Discocotyle* spp. (gill-flukes), *Diplostomum* spp. (the 'eye flukes') and *Acanthocephalus* spp. (spiny-headed worms) can all cause disease in sea-reared salmonids (Brown and Gratzek, 1980; Sedgwick, 1988; Kent, 1992). Infections are mainly due to poor hygiene or in the case of *Acanthocephalus* spp. infections, due to feeding fish with infected raw shrimp or prawn waste. Treatment for these parasites varies from improved hygiene, medicated baths and feeds (Sedgwick, 1988; Kent, 1992). They are not a major problem for sea-reared salmonids.

A serious disease of sea-reared salmonids is that caused by the crustacean parasite *Lepeophtheirus salmonis* (salmon sea-louse) (Sedgwick, 1988; Johnson and Albright, 1992; Johnson *et al*, 1993a). Infestation of sea-farmed Atlantic salmon with this parasite is regarded as one of the most significant of the parasitic diseases affecting these fish (D. Bruno, Pers. Comm.). Infestations result in serious damage to the fish skin and if not treated can lead to significant mortalities from osmotic shock or secondary infections involving *Vibrio* sp. (D. Bruno, Pers. Comm.). The parasites attach anywhere on the body of the fish, but particularly between the vent and tail, causing extensive skin erosion and haemorrhaging in the perianal region (Sedgwick, 1988; Johnson *et al*, 1993a). Generally the prevalence of sea-lice increases with increases in water temperature (Kent, 1992). Treatment of epizootics in Europe is achieved by medicated bath treatment with dichlorvos, alternatively freshwater baths have proved effective (Sedgwick, 1988; Johnson *et al*, 1993a). Recently the use of hydrogen peroxide has been shown to be efficacious (Bruno, 1992a; Johnson *et al*, 1993a).

Diseases of fish caused by algae and dinoflagellates can cause serious losses in the sea-farm environment, as fish cannot escape from the sudden influx of poisonous or damaging material entering their environment (Sedgwick, 1988). Algal blooms can cause reduced oxygen concentrations while some species can also cause damage to fish gills, either mechanically or by producing haemolytic substances (Jameson and Hallegraeff, 1993). Dinoflagellates have caused massive losses of salmonids in sea cages, due to a nerve toxin produced by these organisms (Sedgwick, 1988). Deaths of lingcod, coho, chinook and pink salmon have been caused by high concentrations of the diatoms *Chaetoceros convolutus* and *C. concavicornis*. These diatoms have long spines that penetrate the membranes of the fish causing haemorrhage, osmotic stress and suffocation due to the overproduction of gill mucus (Jameson and Hallegraeff, 1993). Some algae produce haemolysin-like substances that damage the epithelial tissues of the gills, and digestive systems, these algae have been implicated in the in a number of farmed fish kills in North European waters and New Zealand, the latter fish kill resulting in the loss of sea-reared chinook salmon worth \$NZ12 million (Jameson and Hallegraeff, 1993). These blooms cannot be prevented, but monitoring

can help to predict and so forewarn farmers of potentially dangerous blooms, allowing them to minimise the effects, by harvesting the fish early or towing fish to unaffected areas (Jameson and Hallegraeff, 1993). In Tasmania, a nodular gill disease (termed clubbing and necrosis gill syndrome or CNG syndrome) has been observed in sea-farmed Atlantic salmon, resembling amoebic gill disease, and was initially thought to be due to algal blooms (J. Handler, Pers. Comm.). Extensive investigations have failed to find an aetiological agent for this syndrome, and it is now believed that CNG syndrome is not an infectious disease, as no pathogenic organisms were found (Clarke *et al*, 1997).

1.2.5 Diseases common in Tasmania

The Tasmanian salmonid industry is fortunate in that none of the significant diseases that affect overseas sea-farmed salmonids, such as furunculosis, IHNV or infection with the salmon sea louse, have been reported here (AQIS, 1997). However a number of diseases do affect Tasmanian sea-farmed salmonids including vibriosis caused by *Vibrio anguillarum* - a disease common to other sea-farming nations. In Tasmania it has been successfully treated with a locally produced vaccine 'Anguillvac-c' (Munday *et al*, 1992). A nodular gill disease (CNG) has occurred sporadically, which was thought to be caused by algal blooms (J. Handler, Pers. Comm.), but this has not been proved (Clarke *et al*, 1997). Two diseases that do occur in Tasmania are unique in the frequency and severity with which they occur namely amoebic gill disease (AGD) and salmonid cutaneous erosion disease (SCED).

Salmonid cutaneous erosion disease (SCED), caused by the bacterium *Flexibacter maritimus*, caused serious concern to the industry when it first occurred (Carson, 1990; Schmidtke *et al*, 1991). Infection leads to the development of areas of skin necrosis that can be extensive, affecting 25-30% of the body surface (Carson, 1990; Carson *et al*, 1994). When this disease first appeared in Tasmania in 1988, it was severe with losses of fish between 5-20%, and a morbidity rate of 30% (Schmidtke *et al*, 1991). Although severe when it first appeared, the incidence of disease is now quite low, due in large part to improved husbandry, reducing the stress on the fish that

leads to disease. Outbreaks of SCED can be successfully treated with the use of trimethoprim (Cameron, 1991). SCED has been noted in other sea-farmed species in Japan and Europe, but is not a problem for other sea-farming salmonid farming nations, although a similar freshwater version of the disease, caused by *Flexibacter columinaris*, occurs in a number of salmonid sea-farming nations (Carson, 1990). Development of a vaccine for this disease in Tasmania continues to be a priority.

Amoebic gill disease, caused by a *Paramoeba* species, is by far the most significant disease affecting sea-farmed salmonids in Tasmania, occurring in smolt soon after being placed into the sea (Munday *et al*, 1990). AGD recurs throughout the spring, summer and autumn of the smolt's first year in the sea, with most farms reporting at least 3 'waves of infection' (J. Smith, Pers. Comm.). The only successful method of control of AGD is to bath affected fish in freshwater, or tow cages to areas of brackish water (Munday *et al*, 1990). Despite the success of freshwater bathing, AGD has emerged as a major disease problem affecting the relatively young Tasmanian salmonid industry. The major reasons for this being the high cost of freshwater bathing, which is labour intensive and may require the purchase of freshwater, and the threat of major outbreaks affecting more pens than can be treated in time to prevent significant losses (H. King Pers. Comm.). Amoebic gill disease, and related topics will be discussed in detail in the following section.

1.3 Paramoebiasis of aquatic animals

1.3.1 The amoebae

The Protozoa are essentially single-celled, eukaryotic organisms. They are not a natural group of organisms, but have been placed together by taxonomists as a matter of convenience (Levine *et al*, 1980). Amoebae belong to the phylum Sarcomastigophora, subphylum Sarcodina (Levine *et al*, 1980). They are usually naked (lacking a true cell wall), although some form external or internal tests, rigid structures that are either secreted by the cells themselves or composed of foreign material (Weisz, 1973; Brock, 1979; Levine *et al*, 1980). Amoebae move by

extending their cytoplasm outward to form pseudopodia ('false feet'), which are used to engulf food, in a process commonly referred to as phagocytosis. Some amoebae produce flagella, however when present they are usually restricted to the developmental stages. Amoebae can be uninucleate or multinucleate, with most species reproducing asexually by binary or multiple fission. Many form cysts, resting or dormant structures, usually produced as a result of unfavourable environmental conditions such as loss of water and occasionally other noxious agents. (Weisz, 1973; Brock, 1979; Levine *et al*, 1980).

Amoebae are present in all aquatic environments, soils, and generally any environment that contains some moisture. Most are free-living, although some are parasitic, causing disease under certain circumstances. *Naegleria fowleri*, an amoeba with a transient flagellate stage, infects the nasal mucosa and then the central nervous system of young healthy people who have been swimming in thermally polluted water, producing an acute and rapidly fatal meningoencephalitis (Marciano-Cabral, 1988; Mills and Goldsmid, 1995). Several *Acanthamoeba* sp. are responsible for two well defined diseases in humans; chronic amoebic keratitis, usually occurring in healthy individuals who have experienced slight eye trauma or who wear soft contact lenses; and granulomatous amoebic encephalitis (GAE), occurring primarily in chronically ill or immunosuppressed people (Martinez, 1983; Hay *et al*, 1994). More recently another free-living amoeba, *Balamuthia mandrillaris*, has been shown to also cause a fatal GAE in immunocompromised persons (Hua Huang *et al*, 1999).

Entamoeba histolytica is the only proven pathogenic amoeba that is host-bound in humans, causing both intestinal (amoebic dysentery) and extraintestinal disease (the most prevalent being liver abscesses) (Reitano *et al*, 1991; Mills and Goldsmid, 1995). Approximately 12% of the world's population is estimated to be infected, with the disease being most common in developing countries as a result of contaminated water, other high-risk groups include immunocompromised individuals, travellers, and sexually active male homosexuals (Bruckner 1992).

The genus *Paramoeba* belongs to the subclass Gymnamoebia, order Amoebida, family Paramoebidae (Levine *et al*, 1980; Page, 1983). The subclass Gymnamoebia encompasses amoebae that do not produce external or internal tests. Page (1987) reclassified the superclass Rhizopoda, to which the order Amoebida originally belonged, recognising the Rhizopoda as a new phylum, and separating the genus *Paramoeba* into two separate families, Paramoebidae and Vexilliferidae. However, this key does not include detailed descriptions of the different species within the genus, nor does it assign taxonomic positions for some other *Paramoeba* species such as *P. perniciosa* and *P. invadens*. For the purposes of this review and this study, the key provided by Page (1983) has been used, although wherever applicable the revised family and genus assignments are discussed.

Paramoeba species as pathogens have been associated with the viscera and body fluids of a number of marine invertebrates; *P. perniciosa* infects the blue crab (*Callinectes sapidus*), the rock crab (*Cancer irroratus*), and the lobster (*Homarus americanus*), (Sprague *et al*, 1969; Sawyer, 1976). *P. invadens* causes a severe systemic infection in sea urchins, *Strongylocentrotus droebachiensis* (Jones, 1985; Jones and Scheibling, 1985). A *Paramoeba* species has also been described as being associated with a severe gill disease of sea-cultured salmonids, coho salmon, rainbow trout and Atlantic salmon (Kent *et al*, 1988; Roubal *et al*, 1989; Munday *et al*, 1990; Rodger and McArdle, 1996; Palmer *et al*, 1997). A similar disease has been reported in sea-cultured turbot associated with a *Paramoeba* species (Dyková *et al*, 1998c).

1.3.2 *Paramoeba* species

Occurrence of *Paramoeba*

Schaudinn (1896) provided the first written account of the genus *Paramoeba*, in his description of *Paramoeba eilhardi*, a free-living species isolated from a marine aquarium in Berlin. Since then five other species have been described, the free-living *P. aestuarina* (Page, 1970) and *P. schaudinni* (De Faria *et al*, 1922), the free living and parasitic *P. pemaquidensis* (Page, 1970; Cann and Page, 1982; Kent *et al*, 1988), and the parasitic *P. perniciosa* (Sprague *et al*, 1969) and *P. invadens* (Jones, 1985).

Paramoeba species are exclusively marine. Individual species have a wide distribution throughout the world, and are among the most common genera of marine amoebae isolated (Page, 1973).

Since Schaudinn's original description of *P. eilhardi*, the genus has been found in the Mediterranean namely France (Chatton, 1953) and Italy (Grell and Benwitz, 1970) and in the Indian Ocean off Madagascar (Grell and Benwitz, 1970). Another free-living species *P. schaudinni* (De Faria *et al*, 1922), originally described from a sea-water aquarium in Rio de Janeiro, has been reported from the Gulf of Mexico (Sawyer, 1980).

The first parasitic *Paramoeba* species described, *Paramoeba perniciosa*, was isolated from diseased blue crabs (*Callinectes sapidus*) found in Chinoteague Bay and the coast of Virginia, USA (Sprague *et al*, 1969). It is a highly pathogenic, obligate pathogen of the commercially important blue swimmer crab causing 'gray crab disease' (Sawyer, 1969; Sprague *et al*, 1969; Newman and Ward, 1973). All attempts to maintain *P. perniciosa* in culture have been unsuccessful (Sprague *et al*, 1969) and no free-living forms of the species have been reported.

A second parasitic species, *Paramoeba invadens*, was described as the causative agent of periodic mass mortalities of the sea urchin (*Strongylocentrotus droebachiensis*) along the Atlantic Coast of Nova Scotia (Jones, 1985; Jones and Scheibling, 1985). In contrast to *P. perniciosa*, *P. invadens* can be successfully maintained in culture, however studies of water and sediments of Halifax Harbour, Nova Scotia and Canada have yielded no free-living strains of this species (Jellet *et al*, 1989).

Two apparently free-living species of *Paramoeba* were described by Page (1970), *P. pemaquidensis* and *P. aestuarina*, isolated from marine waters around Maine, USA. This constituted the first report of free-living *Paramoeba* species in North America. Page (1973) also reported British strains of *P. pemaquidensis* and surmised that these species were widely distributed throughout the Northern Hemisphere, a theory later verified by Cann and Page (1982). Subsequently *P. pemaquidensis* was

reported in open waters off the Eastern Coast of the USA (Davis *et al*, 1978), in marine sediments off the Western North Atlantic, the Bight of New York and the Gulf of Mexico (Sawyer, 1980), as well as from the shores of North Wales and Western Scotland (Cann and Page, 1982).

More recently *P. pemaquidensis* has been implicated as the causative agent in a gill disease of coho salmon, cultured in sea cages and land based tanks in Washington and California, USA (Kent *et al*, 1988). A *Paramoeba* isolate resembling *P. pemaquidensis* has also been implicated as the causative agent of a similar gill disease in sea-cultured salmonids in Tasmania (Munday, 1988, 1990; Roubal *et al*, 1989), New Zealand (C. Anderson, Pers. Comm.), Ireland (Rodger and McArdle, 1996; Palmer *et al*, 1997) and France (Findlay *et al*, 1995); and also in sea-cultured turbot in Spain (Dyková *et al*, 1998c).

Paramoeba aestuarina is also widely distributed although it is not found as often as *P. pemaquidensis* (Page 1970; Davis *et al*, 1978 and Sawyer, 1980). The first European finding of *P. aestuarina* was reported in 1982 (Cann and Page, 1982). Munson (1992) identified *P. aestaurina* as a common marine amoeba from the coastal waters off Georgia, USA.

Unspeciated isolates of *Paramoeba* have been collected from numerous sites. A species larger than *P. eilhardi* has been isolated from a coastal site around Great Britain (Page, 1978) and *Paramoeba* have also been isolated from sediment cores and sea-water samples from New York Harbour, where the genus appears to have universal distribution in sewage sludge, dredge spoil, and acid waste (Sawyer, 1974).

Characterisation

Characterisation of marine Gymnoamoebae, and therefore *Paramoeba*, relies primarily on morphological and locomotive features. Other characterisation methods such as isoenzyme electrophoresis have not yet been investigated for marine amoebae but may in the future provide resolution of species distinction (Page, 1983).

Locomotive form and size is a valuable marker of amoeba identity, but requires perseverance to acquire useful data. Measurements of length and breadth ratios are also a diagnostic feature, however, Page (1983) recommends caution should be observed in the application of published data in relation to size as some observations suggest that amoebae in culture tend to be smaller than those of the same species in nature. Cytoplasmic inclusions, such as crystals, DNA containing bodies and trichocyst-like bodies; nuclear size, structure and division; floating form; presence of a flagellate stage; cyst formation; physiological features such as euryhalinity, locomotion time, generation time; and fine structure are all useful methods for the identification of marine Gymnamoebae. An excellent and extensive key to the identification of marine Gymnamoebae published by Page (1983) provides a comprehensive key for the identification of free living *Paramoeba* species.

The parasome

The genus *Paramoeba* is characterised by the presence of one or more strongly Feulgan-positive inclusion bodies adjacent to the nucleus. This inclusion body was first described by Schaudinn (1896) and given the name *Nebenkörper* or ‘body close to the nucleus’. This body is characteristic but not unique to the genus, also being present in parasitic amoebae classified in the genus *Janickina* (Chatton, 1953). Chatton called this body ‘nucleus secundus’ or ‘secondary nucleus’, noting its close proximity to the nucleus and concluding that it was a nucleus in its own right. De Faria *et al*, (1922) also considered this body nuclear in type and proposed the name ‘paranucleus’. It has since been known by a number of other terms including ‘Nebenkern’ (Minchin, 1922), and ‘Amphostome’ (Hollande 1940; Chatton, 1953). The terminology was standardised by Page (1970) who proposed the term ‘parasome’ since other terms implied physiological and genetic functions of which there has been

no irrefutable proof to date. Subsequently the term parasome has been almost universally adopted, for the purposes of this review and thesis the term parasome will be used to avoid confusion.

In fixed preparations the parasome is oval or elongate, possessing a strongly Feulgan-positive middle portion sometimes referred to as the *Mittlestück*, and usually two smaller Feulgan-negative end-pieces, the *Endstucke* (Grell, 1961; Grell and Benwitz, 1970; Page, 1970, 1973; Perkins and Castagna, 1971). It is an elongated body with constantly changing orientation, measurements of length and breadth being best taken from living cells by phase contrast, as the parasome has a tendency to shrink in fixed cells (Cann and Page, 1982). Size of the parasome varies with that of the nucleus and the size of the cell (Cann and Page, 1982; Hollande, 1980), and can be used as a morphological feature to help distinguish between species of *Paramoeba*. Although a single parasome is usual, some species have a tendency to have supernumerary parasomes. Parasomes in such species are usually smaller than those where only one or two parasomes are present (Grell, 1961).

Cann and Page (1982) compared the structure of parasomes in a number of *Paramoeba* species (most of which were identified as *P. pemaquidensis*), to the fine structure of *P. eilhardi*, *P. perniciosa* and *Janickina pigmentifera*. They concluded that the parasomes of different species were identical in fine structure, although the parasome of *J. pigmentifera* lacked the close proximity to the nucleus, a characteristic of all species of the genus *Paramoeba*. They supported the nuclear nature of the parasome.

The nature and function of the parasome has not yet been fully elucidated. It is thought to be either an organelle or a symbiont (Grell, 1961; Grell and Benwitz, 1970; Page 1970, 1973; Perkins and Castagna, 1971; Hollande, 1980; Cann and Page, 1982). Hollande (1980) proposed that the parasome in *J. pigmentifera* was a kinetoplastid symbiont he called "*Perkinsella amoebae*".

Grell (1961) in his studies of *P. eilhardi* concluded that the parasome was a true nucleus as it contained strongly Feulgan staining DNA, capable of dividing independently or in synchrony with the amoeba nucleus. He suggested it might, therefore, be the nucleus of a parasite. This view was further supported by Perkins and Castagna (1971) whose studies of the structural features of the parasome revealed the presence of one or two eukaryotic nuclei, nuclear division involving microtubules, the presence of a cytoplasmic component and phagosome formation. They designated the middle region as prokaryotic and the end regions as eukaryotic.

Future studies may elucidate the true nature of the parasome and its relationship to the host, *Paramoeba*. Interestingly, Grell (1961), demonstrated that *P. perniciosa* will die when the parasome is eliminated by UV irradiation, surmising that the relationship may be symbiotic instead of parasitic. The relevance of the parasome, if any, to the pathogenicity of the *Paramoeba* is not known. Its present importance lies in the taxonomy of this genus, the parasome being the most overt distinguishing characteristic of this genus.

Species differentiation

Locomotive forms of the family Paramoebidae appear flattened against the substratum with conical, hyaline projections usually produced from the hyaloplasm (subpseudopodia). The nucleus has a central nucleolus that is close to spherical. The length to breadth ratio of the trophozoite is usually greater than one, for the genus *Paramoeba*. The surface structure is usually highly differentiated and either covered with scales or consisting of a glycocalyx composed of tightly packed apparently tubular elements. Most species of the genus *Paramoeba* have a floating form with fine pseudopodia radiating out from a central mass. (Page, 1970, 1973, 1983; Cann and Page, 1982). Differentiation of the species within the genus *Paramoeba* is determined by these parameters. Table 1.3 has been compiled from the literature to show the main size and morphological differences between the species of the genus *Paramoeba*.

Paramoeba eilhardi is the largest *Paramoeba* with distinct blunt, conical, hyaline subpseudopodia (Cann and Page, 1982). Apart from its large size the other unique feature of this species is the cell surface, which is covered with boat-shaped scales usually 0.35-0.60µm long, consisting of base, rim and 8 or more upright bars (Cann and Page, 1982; Page, 1983). The remaining four species are smaller, with the flattened hyaloplasm occupying 1/5th to 1/3rd of the anterior of the amoeba. No scales are present on the cell surface of these smaller species, the surface being composed of tightly packed apparently tubular elements (Cann and Page, 1982; Page, 1983). *P. eilhardi* grows well in culture, most successfully when diatoms are used as a food source (Cann and Page, 1982). The size and ultrastructural differences of *P. eilhardi* to the other *Paramoeba* species lead to speculation as to the true taxonomic assignment of *P. eilhardi* (Page, 1983), and to a reclassification of this species separating it from some of the other *Paramoeba* species and placing it into the amended family Paramoebidae (Page, 1987).

The smallest *Paramoeba* species described is *Paramoeba aestuarina* (Page 1970). Locomotive forms are observed often with short, blunt, projections from the anterior (moving front) edge of the hyaloplasm, but rarely with prominent longitudinal ridges, a common feature of *P. pemaquidensis*. The species grows reasonably well in culture, but not as well as *P. pemaquidensis* (Page, 1983). The reclassification of this species now places it into the family Vexilliferidae, under the new genus *Neoparamoeba* (Page, 1987).

The most distinctive characteristics of the species *P. perniciosa* are its relatively small size, few linguiform lobopodia, as well as its parasitic nature and failure to survive in common culture media (Sprague *et al*, 1969). *P. perniciosa* has also been described as having a bi-modal size distribution, with small and large forms being observed, with generally the same morphology (Sawyer, 1969; Sprague *et al*, 1969; Johnson, 1977; Couch, 1983). It is not known if these two sizes represent two separate species. The ultrastructure of the surface membrane shows the presence of simple hair-like filaments approximately 0.2µm long (Griffin and Sawyer, 1970). The position of this species in the reclassification is not known.

Jones (1985) was the first researcher to describe *Paramoeba invadens*, the causative agent of periodic mass mortalities of the sea urchin, *Strongylocentrotus droebachiensis*. The anterior region of *P. invadens* is described as often broad, with the hyaloplasm having an irregular margin or short, blunt subpseudopodia sometimes forming linguiform extensions with short pseudopodia. The most distinctive feature of this species is the staining affinity of the parasome, having a Feulgan-negative median segment and Feulgan-positive poles, as opposed to a Feulgan-positive median section described in other species of the genus *Paramoeba*. Radiate floating forms with numerous long pseudopodia, common in most *Paramoeba* species, have not been observed. *P. invadens*, isolated from sea urchins, has been maintained in culture (Jones, 1985), but there is no evidence of a free-living population (Jellet, *et al*, 1989). The position of this species in the reclassification is not known.

Paramoeba pemaquidensis is the most common species of the genus *Paramoeba* (Cann and Page, 1982). The locomotive and floating forms of this species are diverse (Cann and Page, 1982). The presence of one or more longitudinal ridges in the locomotive form, which may continue anteriorly as conical subpseudopodia, are common in this genus (Page, 1983). *P. pemaquidensis* grows readily in culture (Cann and Page, 1982), and unlike *P. perniciosa* and *P. invadens*, free-living species have been described (Page, 1970, 1973; Cann and Page, 1982). The ultrastructure is similar to that described for other *Paramoeba* species but without scales, and some strains of *P. pemaquidensis* showing hair-like filaments extending 200-300nm from the surface, similar to those described for *P. perniciosa*. Like *P. aestuarina*, *P. pemaquidensis* is now classified into the family Vexilliferidae, under the new genus *Neoparamoeba* (Page, 1987), however most publications still refer to the species as *Paramoeba pemaquidensis*.

The *Paramoeba* associated with mortalities of sea-cultured salmonids in the USA has been identified as *P. pemaquidensis* based on the presence of characteristic morphology (Kent *et al*, 1988). Studies of the *Paramoeba* sp. that causes mortalities in sea-cultured Atlantic salmon and rainbow trout in Tasmania also indicate a close

resemblance to *P. pemaquidensis* (Roubal *et al*, 1989; Munday *et al*, 1990), but the authors have not assigned a species name to the *Paramoeba* associated with this disease. More recently, Dyková *et al* (1998c) have identified the causative agent of amoebic gill disease (AGD) outbreaks in turbot, in Spain, as belonging to the genus *Paramoeba*, with the principal characteristics of *P. pemaquidensis*. The authors are reluctant to assign a species name to this *Paramoeba* sp. until non-morphological criteria can be assessed.

These pathogenic species of *Paramoeba* share a number of key characteristics with *P. pemaquidensis*. All three isolates showed the presence of fine hair-like filaments on the surface of the cells associated with damaged gill tissue, have a similar overall cell diameter and size of the parasome and nucleus, all organisms had the ability to thrive in culture - all features consistent with *P. pemaquidensis* (Kent *et al*, 1988; Roubal *et al*, 1989; Munday *et al*, 1990, Dyková *et al*, 1998c). The presence of surface hair-like filaments was not observed in cultured *Paramoeba* by those researchers that investigated the ultrastructure of cultured isolates (Kent *et al*, 1988; Roubal *et al*, 1989; Munday *et al*, 1990). The same groups also reported that cultured isolates were smaller than gill attached organisms. Definitive identification of these isolates as *P. pemaquidensis* may rely on the development of non-morphological methods of identification, such as isoenzyme electrophoresis. Page (1983) views the development of such methods as essential in the species differentiation of genera of the marine Gymnoamoebae.

Page (1983) published a comprehensive key for the identification of free living marine Gymnoamoebae, which continues to be used by researchers investigating AGD in salmonids and turbot, and whilst the reclassification of this group is important from a taxonomic viewpoint, it has little bearing on the study of AGD in fish species. Hence for the purposes of this review and this study *Paramoeba pemaquidensis* and the *Paramoeba* species causing disease in salmonids and turbot were referred to as *Paramoeba* and not *Neoparamoeba*. In addition, the classification of the genus *Paramoeba* as defined by Page (1983) has been used throughout this study, as there is no information regarding the reclassification of other members of this genus, such as *P. invadens* and *P. perniciosa* in the later paper (Page, 1987).

Table 1.3: Distinctive morphological characteristics of the species belonging to the genus *Paramoeba*

	Dimension (µm)*	Mean L:B** ratio	Nucleus (µm)	Parasome (µm)	Super-numerary parasomes	Cell surface
<i>P. eilhardi</i> Ref 1	45-100	NR	≥ 10	≤ 11	Some strains	Boat shaped scales
<i>P. aestuarina</i> Ref 1	< 22	2.0	< 5	≤ 4	Rare	No scales or hairs
<i>P. perniciosa</i> Refs 2-6	<11 (small)*** <25 (large)***	NR	< 5	1-4	Some cells with two	Hairs
<i>P. invadens</i> Ref 7	22-30	≈2.1	3-5	2.5-3	Very rare	No scales or hairs
<i>P. pemaquidensis</i> Refs 1, 8	> 25	1.5-2.0	> 5	> 4	Some strains	Hairs – some strains

* locomotive form
 **Length to breadth ratio
 *** bi-modal size distribution

References: 1. Page, 1983
 2. Sprague *et al*, 1969
 3. Sawyer, 1969
 4. Johnson, 1977
 5. Couch, 1983
 6. Griffin and Sawyer, 1970
 7. Jones, 1985
 8. Cann and Page, 1982

1.3.3 Diseases of aquatic animals caused by amoebae

While amoebae are generally free-living, some are undoubtedly parasitic and the cause of pathological conditions, and others are likely to be commensals. Historically there have been few reports regarding amoebic infection in aquatic animals, most likely due to a lack of economic reason rather than an absence of parasitic and commensal amoebae. Most of the reports of amoebae isolated from aquatic animals are from fish cultured in freshwater facilities. The following reports have been divided into those that cause gill disease; those that cause systemic disease; those that affect both the gills and systemic systems; and those reports that show no pathological changes attributed to the isolated amoebae. A disease of cockles, caused by an amoeba, is also discussed. An extensive search of the literature has failed to find any other reference to amoebae isolated from other aquatic organisms, except those relating to paramoebiasis of sea urchins and crab (both of which will be discussed in detail later in this review). Paramoebiasis of sea-cultured fish will also be discussed in detail later in this review.

Amoebae associated with gill disease

Chatton (1909, 1910) was the first to describe a gill disease associated with a Vahlkampfid amoebae (*Amoeba mucicola*), in marine labrids (*Symphodus melops* and *S. tinca*), the amoebae were described as being so numerous that they resembled an epithelial sheet. Sawyer *et al* (1974, 1975) observed mortalities in rainbow trout, coho salmon and chinook salmon fingerlings reared in freshwater hatcheries in the US, associated with the normally free-living amoeba *Thecamoeba hoffmani*. Histological examination of the gills showed cellular proliferation of the gill tissue adjacent to amoebae, short attenuated gill tissue and cellular infiltration at the base of the lamellae. Chloride cell hypertrophy was also apparent. The authors suggested that mortality was due to respiratory impairment and asphyxiation, due to the presence of the amoebae in the interlamellar spaces. Severe mortalities at a hatchery in Michigan subsided when fish were exposed to formalin, 1:5,000 for one hour.

A unique form of proliferative gill disease, termed 'nodular gill disease' (NGD), was observed in rainbow trout fingerlings in Canada, associated with amoeba-like angular 'A'-cells (Dauost and Ferguson, 1985). The lesions on the gills of affected fish were distinctive, showing white nodules 1-2 mm in diameter in the distal regions of the gill filaments, often with a diffuse white discoloration of the branchial arches.

Histologically, the gill lesions were multifocal among the filaments, severe in the distal half of the filaments, frequently resulting in filament fusion and the formation of nodules; little necrosis of surface epithelial cells was observed. The distinctive 'A'-cells were associated with the nodules, confined to the epithelial surface of the hyperplastic masses, sometimes in layers of two or three. The 'A' cells were relatively small, the largest measuring 12.61µm and the smallest 4.85µm. Numerous mucous cells were present on the surface and within affected tissue. The 'A'-cells were not found to be invasive, leading the authors to propose that parasitic chemicals were important rather than mechanical damage. Morbidity and mortality data were not included in this report, although it was noted that this disease was not observed as frequently as bacterial gill disease (BGD), associated with the bacterium *Flavobacterium branchiophilus*.

A secondary amoebic gill disease (AGD) was described in juvenile rainbow trout, spontaneously infected with bacterial gill disease (BGD), in a freshwater recirculation system (Bullock *et al*, 1994). Bacterial gill disease occurred within 6-8 days after the fish were placed in the recirculation system; with amoebae appearing on day 9 and continuing to increase in number to day 15. Histopathology of AGD infected gills showed large numbers of amoebae, 10µm in diameter with a vesiculate nucleus and prominent endosome, on the surface of the gills associated with severe hyperplasia. BGD was successfully treated with one or more treatments with chloramine-T (9-15mg/L). However this treatment failed to control the amoeba infection, resulting a mortality rate of 71-91%. Subsequently, a treatment with formalin (167mg/L) for one hour was found to control the amoeba infection. The amoeba involved in this outbreak was not cultured or identified.

Noble *et al* (1997), reported a similar case of recurrent AGD in rainbow trout, cultured in a freshwater recirculation system. Macroscopically, the gills of infected fish had white inflamed tips, covered in mucus. Histopathology showed many amoebae associated with areas of hyperplasia and lamellar fusion. The amoebae were small (10µm in diameter), each with a vesiculate nucleus and prominent endosome, as described by Bullock *et al* (1994). The organism was also thought to resemble the A-cells described by Daoust and Ferguson (1985). Based on morphology the authors concluded that the amoebae appeared most related to the family Cochliopodiidae. Treatment with formalin, 167 parts per million (ppm) for one hour, eliminated the amoebae from the gills. Subsequent treatments of the entire system with 50-167 ppm reduced the intensity of further infections. Attempts to culture the amoeba were not successful.

More recently NGD has been reported in Arctic charr (*Salvelinus alpinus*) from a commercial farm in eastern Canada; the first report of NGD in a species other than rainbow trout (Speare 1999). Speare reported a mixed infection of the gills with *Flavobacterium branchiophilum*, the causative agent of bacterial gill disease, and amoebae similar to those reported responsible for NGD in rainbow trout (Daoust and Ferguson, 1985; Noble *et al*, 1997). Gill pathology was similar to the reported cases of NGD in rainbow trout (Daoust and Ferguson, 1985; Noble *et al*, 1997). Attempts to treat this mixed infection of NGD and BGD with formalin baths, one of the successful treatments for BGD, often resulted in severe mortalities during or after treatment, suggesting this treatment was inappropriate for mixed infections.

Infections of heavy and medium intensity with an unidentified cochlipodid amoeba were reported in small numbers of juvenile rainbow and brown trout, from wild populations in upper Colorado (Schisler *et al*, 1999). This study of protozoan gill ectoparasites was conducted to determine the health of wild salmonids, and the role of parasites in the decline or complete loss of year classes of rainbow trout in the region. The authors believe the unidentified amoeba to be a free-living species that

infests fish at suboptimal water quality conditions; most probably high water temperatures and low water flow. The authors concluded that the gill ectoparasites, including the amoeba, most likely contributed to the loss of individual fish, but were not high enough to explain the total collapse of year-classes observed.

Amoebae associated with systemic disease

Voelker *et al* (1977) reported several cases of systemic amoebiasis in goldfish (*Carassius auratus*), kept in aquariums. Affected fish were lethargic, with abdominal distension and showed loss of appetite. Granulomas containing amoebae were found in many organs, but were most numerous in the kidneys. The presence of the organism in so many tissues suggested that it might be spread through the blood. The amoebae were small, ranging from 2.4-4.6µm in diameter; and based on mitotic stages¹ it was suggested that they belong to the family Hartmannellidae, although the surface structure was most similar to *Acanthamoeba castellani*. Amoebae were also observed in clinically normal fish, and it is possible that the goldfish may carry the infection for life with death occurring only when the fish are suddenly stressed. The organism was unable to be cultured. More recently, Dyková *et al* (1996) found granulomatous inflammatory lesions associated with the presence of amoebae in 59% of goldfish examined, but failed to culture or identify the causative amoeba.

Serious seasonal epizootics of systemic amoebiasis were reported in hatchery-reared rainbow trout from Italy (Sawyer *et al*, 1978). Amoebae were found in lesions and nodules of the kidney, spleen, liver and peritoneum. The disease was transmitted to naïve fish by feeding them with infected minced kidney tissue. The amoeba was identified as *Vexillifera bacillipedes*, a free-living mayorellid amoeba, and described by the authors as “amphizoic” (capable of existing as free-living or as parasitic organisms). *In vitro* studies demonstrated the ability of this freshwater amoeba to survive and multiply in brackish water (5-10 ppt); but it was unable to multiply in sea-water medium. No morbidity and mortality data were included in this report.

¹ Members of the family Hartmannellidae divide by true mitosis, as opposed to amoebae of the family Schizopyrenidae (Valkampfidae) which divide by promitosis.

Amoebae associated with gill and systemic disease

Amoebae were thought responsible for two large fish kills of blue tilapia (*Sarotherodon aureus*), in a lake and experimental pond in the US, in 1969 and 1971 (cited Taylor, 1977). Amoebae were found in the peritoneal fluid, intestinal mucosa, and on the gills. Histopathology of the gills showed severe erosion but no inflammatory response. One isolate was tentatively identified as *Acanthamoeba* sp. Taylor (1977) screened 23 species of freshwater fish from nine south eastern states in the US for the presence of small free-living amoebae. Eighteen isolates of amoeba were obtained from the gills and internal organs of 11 species of fish. A *Naegleria* sp. and four isolates belonging to the genus *Vahlkampfia* were isolated from organs having direct contact with the aquatic environment. No pathological changes been reported in fish associated with these genera, and they were thought to be facultative dwellers that became trapped in the mucus. *Acanthamoeba polyphaga* was isolated from numerous organs in the fish including the gills, blood and spleen, and interestingly is one of the species of *Acanthamoeba* known to cause corneal ulceration and blindness in humans (Martinez, 1983; Hay *et al*, 1994). The ability of *A. polyphaga*, isolated from the spleen of a bass, to infect and produce a systemic infection in blue tilapia and carp (*Cyprinus carpio*), was also demonstrated, suggesting that the amoeba responsible for the fish kills in 1969 and 1971 was *A. polyphaga*.

Nash *et al* (1988) described a severe proliferative gill and systemic disease in European catfish (*Silurus glanis*) cultured in a recirculation system in Germany, associated with a parasite resembling amoebic trophozoites. Mortality rates of up to 30% were recorded. Histopathology showed the amoebae present in large numbers in the gills, and the renal haematopoietic tissue, liver, intestine and spleen. Histopathology of the gill tissue showed extensive and diffuse epithelial cell hyperplasia, with almost total secondary and multifocal primary lamellar fusion, and multi-celled layers of mucous cells. The amoebae were between 7.5-15µm in diameter, with prominent nucleoli; preliminary identification suggested an *Acanthamoeba* sp. The authors considered poor environmental conditions and high levels of bacteria in the water as essential for initial gill infection, and with undetermined stress factors contributing to the host's inability to limit the internal

spread of the parasite. Infections occurred when the water temperatures were between 24-26°C, which unfortunately is the temperature required for optimal growth of cultured catfish. Lytic enzymes were also suggested as a factor in tissue necrosis and inflammatory response.

Amoebae not associated with pathological changes

Davis (1926) reported the first published account of amoebae present in farmed salmonids. Amoebae were observed in the stomach and intestine of hatchery-reared (freshwater) salmonids (species names unreported) in the US. This cyst-forming amoeba was abundant in the mucus covering the epithelial lining of the stomach, with trophozoites only found in the stomach and cysts found in the intestine. The author observed some unique characteristics of the encysted stage and proposed a new genus *Schizamoeba* gen. nov. and species, *Schizamoeba salmonis* sp. nov. There was no evidence that the amoeba was directly harmful to the hosts, however it was suggested that they might be important in consuming nutrients important for optimal growth of the salmonids. There have been no other published reports regarding this amoeba in salmonids.

A single amoeba was observed on the gills of an amago, *Oncorhynchus rhodurus*, although its taxonomic identity and pathogenic role, if any, were not determined (Kubota and Kamata, 1971).

Dyková *et al* (1996) isolated 3 strains of amoebae from the organs of goldfish, one identified as *Vannella platypodia* and the other two as *Rosculus ithacus*. This report constituted the first report of these species in the organs of fish. Dyková *et al* (1998a) also reported the isolation of 4 strains of non-encysting amoebae from the organs of freshwater fish, demonstrating the ability of amoebae to infect fish. *Vannella platypodia* was isolated twice, from the kidney tissue of the common goldfish, and from the brain of a chub (*Leuciscus cephalus*). *Vexillifera expectata* was isolated from the liver of a perch (*Perca fluviatilis*). The fourth strain was isolated from the spleen of a bullhead (*Cottus gobio*), and could not be identified. No pathological changes were reported associated with these amoebae.

Three amoeba species were isolated from three farmed Nile tilapias, *Hartmannella vermiformis* and *Rosculus ithacus* from the kidney tissue, and an isolate sharing morphological characteristics of *Mayorella* and *Platyamoeba* spp. from the liver (Dyková *et al*, 1997). The amoebae were not associated with systemic amoebiasis or losses; however pathogenicity of *H. vermiformis* was proved in two fish hosts.

A “scale”-bearing amoeba *Cochliopodium minus* was also isolated from the gills and other organs of perch (Dyková *et al*, 1998b). The ability of this amoeba to colonise the gills and internal organs of another fish species, *Heteropneutes fossilis*, a bottom-dwelling fish, was proved experimentally by keeping the fish in water enriched with trophozoites and cysts from cultured *C. minus*. It is unclear whether the authors believe this organism to be responsible for disease, as no pathological changes or histopathology were reported associated with the amoeba.

Amoebae in other aquatic animals

Large numbers of an unidentified amoeba were observed in the subepithelial tissues of the gills of the cockle, *Cerastoderma edule*, taken from the estuarine region of Aveiro, Portugal (Azedo, 1997). The amoebae were discovered by chance during a study of the life cycle of a microsporidian hyperparasite (*Unikaryon legeri*) in the cockle. Histopathology associated with the amoeba revealed haemocytic infiltration and necrosis cells. The cockles sampled were described as gaping and moribund, with 41% being infected with amoebae and only 26% being infected with both the amoebae and microsporidians. The author believed that mortality of the cockle was a result of amoeba activity in the gills rather than the microsporidian hyperparasite.

1.3.4 Paramoebiasis of crabs

In 1966 Sprague and Beckett described a disease accompanying mortalities of the blue crab (*Callinectes sapidus*) in shedding tanks, in Eastern Maryland and Virginia in the US. The disease was named 'grey crab disease' due to the greyish discoloration of the ventral surface of the crab. Fisherman in Maryland and Virginia, had frequently noted this disease, due to the distinctive grey appearance of infected crabs (Couch, 1983). The range of the disease appears to be limited between Maryland and North Carolina (Sawyer, 1969; Newman and Ward, 1973). Sawyer (1976) identified the rock crab, (*Cancer irrotatus*) and the lobster (*Homarus americanus*) as additional host animals, although both crustaceans appeared to have a strong defence against the invasive amoebae. There have been no reports of gross signs of disease or mortality in rock crabs or lobsters.

The disease was attributed to infection with a *Paramoeba* species, later named *Paramoeba pernicioso*, as grey crabs always contained very large numbers of these amoebae (Sprague and Beckett, 1966, 1968; Sprague *et al*, 1969; Newman and Ward, 1973). Mass mortalities and chronic low level losses of blue crabs due to *P. pernicioso* is of special concern because of the economic value of these shellfish (Sawyer, 1969; Couch, 1983).

Characteristics of the disease

Moribund crabs exhibit a grey discoloration of the ventral surface or abdomen, are sluggish, show a cloudy grey discoloration of the haemolymph and watery tissue (Sawyer, 1969; Sprague *et al*, 1969; Newman and Ward, 1973; Johnson, 1977). This greyish discoloration is only observed in crabs with terminal infection, however not in all crabs with terminal infections (Johnson, 1977). Large numbers of amoebae are found by microscopy in the haemolymph of crabs prior to death, sometimes completely replacing the blood cells (Sprague and Beckett, 1968; Sprague *et al*, 1969). Histological examination of muscle tissue shows disintegrated tissue thought to be caused by unknown lytic properties of the parasite and in heavy infections, the haemal spaces of the crab are filled with amoebae which appear to impinge on a variety of tissues (Sprague *et al*, 1969). Sawyer (1969) observed that heavily infected

crabs had amoebae in the vascular channels but organs and tissues were not infected. Light infections had amoebae in the intestinal wall only surrounded by haemocytes or tissue cells, which according to Sawyer suggested a tissue response.

Johnson (1977) examined the major organs and tissues of the blue crab for the presence of *Paramoeba* in all stages of infection. *Paramoeba* were rarely seen in the gills and hepatopancreas until the disease was advanced; the heart contained no amoebae in light infections and rarely in moderate to heavy infections, but they were always present in terminal infections. The antennal, midgut and Y-gland nearly always contained amoebae, however few crabs showed amoebae in the endothelium of the blood vessels regardless of the severity of the disease. Johnson (1977) postulated that “by their patterns of tissue distribution amoebae may avoid host recognition as well as occupy nutritionally adequate areas”.

A decline in haemocytes and a reduced ability of the crab serum to coagulate has been noted in crabs infected with *P. perniciosus* (Sawyer *et al*, 1970; Pauley *et al*, 1975). Comparison of healthy and infected crabs by immunoelectrophoresis and acrylamide gel electrophoresis showed a marked reduction in protein, glucose and copper levels in the serum of infected crabs (Pauley *et al*, 1975). The host response to infection is often massive and involves humoral responses (Johnson, 1977). Crab cells attempt to phagocytose the amoebae and occasional attempts at encapsulation are found in early and intermediate infection (Couch, 1983). The precise cause of death by *P. perniciosus* is not known.

Characterisation of the pathogen

As described in Section 1.3.2 - species differentiation.

Epizootiology

Epizootiological studies of this disease have shown the peak prevalence and mortality to occur during spring and summer (Sawyer, 1969; Newman and Ward, 1973, Johnson, 1977). Prevalence and mortality rates appear to be variable from year to year. A mortality rate of 20-30% was observed in a study of blue crabs in a shedding tank on the eastern shore of Maryland in 1965 (Sprague and Beckett, 1966). Sawyer (1969) studied pre-molt (peeler) and inter-molt (hard) crabs from Chincoteague Bay, Virginia, during the summer of 1968 and found that 35% of pre-molt and 8% of intermolt crabs were positive for *P. perniciosus*. Newman and Ward (1973) found the peak prevalence of 17% during an outbreak in the same area, with data suggesting a 100% mortality of infected crabs. Johnson (1977), during a study in Chincoteague Bay, found the prevalence of *P. perniciosus* in tissues to be at a peak of 57% in July, with prevalences throughout the rest of the year being between 6-22%. Couch (1983) reported an epizootic of *P. perniciosus* in hibernating blue crabs during the winter months of 1968 and 1969, representing a new seasonal occurrence. In contrast Sawyer (1969) failed to find amoebae in hibernating crabs sampled during the winter. Johnson (1977) found that terminal disease appeared to coincide with water temperatures above 13°C, although no definitive investigation of water temperature and disease incidence has been undertaken.

How *P. perniciosus* causes infection and disease is not known. It was suggested that as the infected crabs die from infection they cannot be considered a natural reservoir of infection (Newman and Ward, 1973). However Johnson's (1977) studies of the parasite distribution in the crab suggested that for transmission to occur the host must die, disintegrate or be eaten prior to the death, to release the amoebae. Successful establishment of disease by inoculation suggests that wounds may also provide a portal of entry for the parasite. The seasonality of the disease correlates with the summer molting period, which suggests that the amoebae may enter the soft crabs through lesions in their cuticles (Couch, 1983).

Diagnosis

Terminal infection is easily diagnosed when amoebae are present in circulating blood. Amoebae are best observed in haemolymph smears fixed in Bouin's, Davidson's, Hollande's or 10% formalin solutions, and stained with iron haematoxylin or Giemsa (Sawyer, 1969; Newman and Ward, 1973; Johnson, 1988). Amoebae can also be observed in the haemolymph by phase contrast microscopy (Johnson, 1988). These methods allow the identification of the parasome, an identifying characteristic of the genus *Paramoeba*. In heavy infections, before the appearance of amoebae in circulating blood, amoebae can be observed by phase microscopy in squashes of subepithelial connective tissue (Johnson, 1988).

Isolation of the pathogen

All attempts to maintain *P. perniciosus* in culture have been unsuccessful (Sprague *et al*, 1969).

Pathogenicity

The presence of large numbers of *Paramoeba* species in the circulatory system of *Callinectes sapidus*, suffering from 'grey crab' disease was the first indication that this species could be pathogenic (Sprague and Beckett, 1966; Sawyer *et al*, 1969). Later, Newman and Ward (1973) observed that all animals with overt infection eventually died. 'Grey crab' disease was successfully transmitted to healthy crabs by the inoculation of haemolymph from infected crabs into the intersegmental membranes that separates the most posterior leg from the carapace (Johnson, 1977). *P. perniciosus* is known to have a bi-modal size distribution with small and large forms being observed (Sawyer, 1969; Sprague *et al*, 1969). In the transmission experiments conducted by Johnson (1977) small-form amoebae were used, the resulting infection resembled that acquired naturally, small-form amoebae predominating (Johnson, 1977). The author

postulated that the production of small forms may be an essential part of the life cycle of *P. perniciosus*, as they occur regularly in terminal infections. Transmission of disease using cultured *P. perniciosus* has not been achieved due to the failure of this species to be maintained in culture (Sprague and Beckett, 1969).

Exactly how the amoebae cause death in the crabs is not fully understood.

P. perniciosus is a parasite of connective tissue, haemal spaces and haemolymph. Histology preparations show disintegration of the muscle tissue and blood cells that may be due to lytic properties of the parasite (Sprague *et al*, 1969; Sawyer *et al*, 1970). The amoeba is not an intracellular parasite but histological sections often show large halos surrounding amoebae supporting the hypothesised lytic properties of the parasite (Couch, 1983). It has been suggested that the presence of amoebae in the circulatory system of the crab may suggest an unusual host-parasite relationship that may involve a cryptic tissue phase of growth (Sawyer, 1969). Newman and Ward (1973) suggested that some crabs may be able to contain the infection before signs of overt disease are observed. Pauley *et al* (1975) found that serum protein and copper levels decline in relation to the severity of disease, suggesting that the hosts may not be able to adequately compete for their own nutrients; loss of fibrinogen as part of the total serum protein may also explain why the haemolymph in infected crabs fails to clot. Low levels of haemocyanin in infected crabs indicate there are probably insufficient oxygen levels in the host, due to induced anaemia. Death may be due to a combination of insufficient oxygen and nutrients (Pauley *et al*, 1975).

Control

There have been no published attempts to treat or control this disease.

1.3.5 Paramoebiasis of sea urchins

There have been many reports in the last two decades of mass mortalities of various species of sea urchins, attributed to disease, occurring across the globe (Miller and Colodey, 1983, Scheibling and Hennigar, 1997). Microbial pathogens were suspected, but only in mass mortalities of the sea urchin (*Strongylocentrotus droebachiensis*), in Nova Scotia, has a specific microorganism been identified. Mass mortalities of this sea urchin were reported occurring along the Atlantic Coast of Nova Scotia from 1980 to 1983, and laboratory studies showed that a waterborne infective agent was involved (Miller and Colodey, 1983; Scheibling and Stephenson, 1984). Amoebae, morphologically similar to the genus *Paramoeba* were identified in the tissues of urchins showing signs of disease (Scheibling, 1984). This infective agent was later identified as a new, *Paramoeba* species and given the name *Paramoeba invadens* (Jones, 1985). Since then regular epizootics have occurred and been reported in the literature (Scheibling and Hennigar, 1997). This disease has important implications for the rapidly expanding sea urchin fishery in Nova Scotia, which is expected to periodically crash; and also to the community structure as barren grounds, previously dominated by sea urchins, are colonised by kelps and macroalgae (Scheibling and Hennigar, 1997).

Characteristics of the disease

The gross signs of paramoebiasis in sea urchins include muscle degeneration in the tube feet, spines and mouthparts, which result in loss of attachment capability, cessation of feeding, immobility and eventual death (Scheibling, 1984; Scheibling and Stephenson, 1984; Jones and Scheibling, 1985; Jones *et al*, 1985). Due to their inability to attach and the loss of defensive spines, infected sea urchins suffer from increased predation (Scheibling, 1984). Bacterial infection in late stage paramoebiasis, in both naturally and experimentally infected urchins, is common, occurring after loss of attachment in moribund individuals, the bacteria being thought to be secondary invaders in moribund urchins (Jones and Scheibling, 1985; Jellet *et al*, 1988).

Histologically, both nerve and water vascular tissues show signs of disease and contain amoebae, either in small clusters or scattered singly (Jones and Scheibling, 1985). Signs of the disease are most apparent in the body wall and associated tissues, and also in the coelomic fluid (Jones *et al*, 1985). Extensive degeneration of the muscle and connective tissue of the spine base, tube feet and ampullae is observed in diseased echinoids (Jones *et al*, 1985). *P. invadens* is widespread in the tissues but generally at low density (Jones and Scheibling, 1985). Nerve tissue is often heavily infiltrated with red spherule cells (a type of coelomocyte). Amoeba infected water vascular tissues show considerable disintegration. The epithelium and coelomic lining cells of the tube feet and ampullae show evidence of sloughing and the muscle layers show signs of fragmentation.

Infected urchins have pinkish grey coelomic fluid (Jones and Scheibling, 1985; Jones *et al*, 1985). Quantitative changes in the coelomic fluid have been reported in experimentally infected urchins (Jellet *et al*, 1988). Elevated protein levels were found in infected urchins, being about twice that of uninfected urchins, and it was suggested that this may be a result of an amoeba-induced autolytic enzyme secreted by the echinoid cells, or a result of tissue or coelomic cell lysis. Alternatively it could be a humoral immunity factor, although no resistance to the disease was noted. At 16°C, the total number of coelomocytes, (cells found in the coelomic fluid, such as phagocytes, thought to be involved in food transport, waste elimination and defence), were reduced in infected urchins. Phagocyte numbers were normal, the reduction in infected urchins being mainly due to a decline in white spherule and vibratile cells.

The host response has not been extensively studied, however Jones and Scheibling (1985) reported no signs of phagocytosis, lysis or encapsulation or encapsulation of the *Paramoeba* by the host. Normal phagocyte numbers in the coelomic fluid of infected urchins may indicate a failure of these cells to recognise the invading amoebae as foreign, or perhaps the replacement of phagocytes to exactly compensate for losses from the coelomic fluid (Jellet *et al*, 1988).

Characterisation of the pathogen

As described in Section 1.3.2., species differentiation.

Epizootiology

Mortalities of sea urchin usually occur with periods of unusually high sea-water temperatures, in late summer and autumn (Miller and Colodey, 1983; Scheibling, 1984; Scheibling and Stephenson, 1984; Scheibling and Hennigar, 1997). High mortality rates were observed when water temperatures exceeded 15°C, but subsided when temperatures fell below 8°C (Scheibling and Stephenson, 1984; Scheibling and Hennigar, 1997). Laboratory studies showed that transmission and progression of the disease was temperature dependant (Scheibling and Stephenson, 1984; Scheibling and Hennigar, 1997). *In vitro* growth characteristics of *P. invadens* reflect the seasonality of infection. Maximal growth occurs at 15-20°C, but is much reduced at 10-12°C and appears to cease at 2-5°C. *P. invadens* did not survive in monoaxenic culture at 27°C (Jellet and Scheibling, 1988b).

To date *P. invadens* has only been isolated from infected tissues of sea urchins, but not from the sea or sediments of the urchin habitat, suggesting that infection is not due to an endemic population (Jones, 1985; Jellet *et al*, 1989). Jones and Scheibling (1985) postulated that the *Paramoeba* species may be local in origin and cause disease only when the sea urchin resistance was decreased due to high water temperatures. Alternatively the pathogen may be exotic and introduced by warm water currents. Recently, outbreaks of paramoebiasis have been observed associated with increased coastal warming (Scheibling and Hennigar, 1997). The authors also found a positive correlation between disease outbreaks and relatively high tropical storm and hurricane activity in the northwest Atlantic. They suggest, “large-scale oceanographic and meteorological events may play an important role in triggering epizootics by transporting the infective agent and/or creating environmental

conditions conducive to the propagation of the disease". However, Jellet and Scheibling (1988a) postulated that the extinction of *P. invadens* in monoaxenic culture at 27°C indicates that it is unlikely to originate from tropical warm water masses. *P. invadens* appears to be relatively host specific, and there is no evidence that co-occurring echinoderms are affected (Scheibling and Stephenson, 1984).

Diagnosis

The disease appears to be easily diagnosed by examining the coelomic fluid, or squashes of radial nerves and ampullae, from sea urchins with signs of the disease, for the presence of *Paramoeba*, by bright field, phase contrast or Nomarski interference-contrast microscopy (Jones, 1985; Jones and Scheibling, 1985).

Isolation of the pathogen

In vitro cultures are routinely established from radial nerve fragments taken from sea urchins showing signs of disease. *P. invadens* has been successfully maintained on malt-yeast-75% seawater agar (Page, 1973), with a sterile seawater overlay (Jones and Scheibling, 1985). The amoeba also grows well on 0.6% non-nutrient agar in 100% artificial seawater (Jones, 1985; Jellet and Scheibling, 1988a,b). In both culture systems marine bacteria isolated with the amoeba, or isolated from the surface of the sea urchin, are used as a food source. *Pseudomonas nautica* has been successfully been used by some researchers as a monoaxenic food source, suppressing the natural marine bacterial flora with the antibiotics penicillin and streptomycin (Jellet and Scheibling, 1988a,b).

Pathogenicity

Initial evidence that a *Paramoeba* species was responsible for mass mortalities of the sea urchin, came from its presence in sections of tissue from diseased urchins (Jones *et al*, 1985a, b; Jones and Scheibling, 1985). No other apparent pathogens were observed in these tissues, although bacterial invasion was noted in moribund individuals (Jones *et al*, 1985). Laboratory studies undertaken showed that a water-borne infective agent was involved in the disease and that transmission and

progression of the disease was temperature dependant (Scheibling and Stephenson, 1984). Subsequent experiments have shown that cultured *P. invadens* from diseased tissues produced signs of disease identical to naturally infected urchins when injected into the coelom of healthy urchins (Jones and Scheibling, 1985). Infection via water-borne routes also caused infection (Scheibling and Stephenson, 1984; Jones and Scheibling, 1985). Pathogenicity tests have shown a loss of virulence in monoaxenic cultured *P. invadens* after 15 weeks and in polyaxenic culture after 58 weeks; the authors suggest that periodic passage through the sea urchins may be required to maintain virulence (Jellet and Scheibling, 1988).

The method of pathogenicity of *P. invadens* is unclear as amoebae are often sparsely distributed throughout host tissues (Jones *et al*, 1985). A decrease in the coelomocyte number has been observed in urchins kept at 16°C, indicating that prolonged periods of relatively high temperatures may be stressful and a predisposing factor for infection (Jellet *et al*, 1988). This factor, combined with the increased growth rate of *P. invadens* at temperatures between 15-20°C (Jellet and Scheibling, 1988), may in combination increase the likelihood of infection (Jellet *et al*, 1988). Infected urchins also show a significantly higher protein concentration in cell free coelomic fluid, possibly due to secretion of autolytic enzymes by the echinoid cells in response to the pathogen (Jellet *et al*, 1988). These changes may explain the histopathological damage seen in infected animals (Jones *et al*, 1985). Mechanisms such as cytolysis, damage to nerve tissue, inhibition of host defences, and secondary bacterial invasion of the coelomic fluid in the late stages of disease, may contribute to the eventual death of the urchin (Jones, 1985).

Control

There have been no published attempts to treat or control this disease.

1.3.6 Paramoebiasis of salmonids and turbot

The first cases of amoebic gill disease (AGD) in marine fish, attributed to a *Paramoeba* sp. occurred in 1985, in sea-cultured coho salmon reared in net pens and land based tanks in Washington and California (Kent *et al*, 1988). A mortality rate of 25% was observed in the net pens in 1985; the disease recurred in the autumns of 1986 and 1987 but with a lower morbidity and mortality rate (Kent *et al*, 1988). Since then outbreaks have been sporadic (M. Kent, Pers. Comm.). Morphological characteristics indicated that the amoeba was most closely related to the free-living *P. pemaquidensis* (Kent *et al*, 1988).

A similar disease of unknown aetiology was described in sea-farmed Atlantic salmon and rainbow trout in Tasmania, soon after the commencement of sea-cage farming in 1984 (Roubal *et al*, 1989; Munday *et al*, 1990). Mortality in these fish was variable, reaching 2% per day, and up to 50% in untreated cases (Munday *et al*, 1990). Studies of this *Paramoeba* sp. indicated a close relationship with *P. pemaquidensis*, however a species name was not assigned. This disease has emerged as a major health problem for the salmonid industry in Tasmania with most farms each year reporting at least 3 waves of infection in smolt during spring, summer and autumn (J. Smith, Pers. Comm.).

Sporadic outbreaks of AGD caused by *Paramoeba* spp. have since been described in: sea-cage farmed chinook salmon in New Zealand (C. Anderson, Pers. Comm.); in sea-cage farmed Atlantic salmon in Ireland, where mortality rates are variable ranging from no mortality in some farms to greater than 10% in others (Rodger and McArdle, 1996); and in sea-cultured turbot in Spain, where mortalities of up to 20% were observed in some tanks (Dyková *et al*, 1998c). In France, AGD caused by *Paramoeba* sp. has emerged a major threat to Atlantic salmon culture, and to a lesser extent the culture of rainbow and brown trout (Findlay *et al*, 1995).

Characteristics of the disease

The pathological changes of AGD, caused by *Paramoeba* spp., in sea-cultured salmonids and turbot is confined exclusively to the gills of the fish. The disease is never systemic (Kent *et al*, 1988; Roubal *et al*, 1989; Munday *et al*, 1990; Dyková *et al*, 1998c).

Diseased fish show typical signs of respiratory distress, appearing sluggish with open operculae (Munday *et al*, 1990), often accumulating at the surface or corners of the pens (Kent *et al*, 1988), or swimming ventral side up (Dyková *et al*, 1998c). There is often some depression of appetite, but this has been described as variable with dead fish often in good condition with food in their stomachs (Munday *et al*, 1990; Dyková *et al*, 1998c).

Macroscopically the gills of affected fish show the presence of excessive mucus ((Munday *et al*, 1990, 1993; Dyková *et al*, 1998c). In Atlantic salmon this mucoid branchitis can be patchy, or relatively diffuse in rainbow trout (Munday *et al*, 1990). The mucus is relatively diffuse on the gills of rainbow trout, but in Atlantic salmon the lesions take the form of discrete patches (Munday *et al*, 1990). Dyková *et al* (1998c) also described clubbing of the gill filaments, some of which were shortened and necrotic. The presence of mucous patches on the gills of sea cultured Atlantic salmon is commonly used in Tasmanian sea farms as the primary diagnostic characteristic to identify AGD (Alexander, 1991). The fish suffer from anoxia and respiratory distress, due in large part to a reduction of the surface respiratory area of the gills, leading to death as a result of respiratory failure (Munday *et al*, 1990; Dyková *et al*, 1995).

Histologically the most outstanding feature of AGD in salmonids and turbot is epithelial hyperplasia involving the secondary lamellae. This hyperplasia leads to clubbing and often complete or partial fusion of the secondary lamellae (Kent *et al*, 1988; Roubal *et al*, 1989; Munday *et al*, 1990; Dyková *et al*, 1995). Sometimes this fusion results in the formation of large vesicles or cysts, often containing amoebae (Kent *et al*, 1988; Munday *et al*, 1990; Dyková *et al*, 1995). Dyková *et al* (1998c) described the progression of the damage to the secondary lamellae. Initially amoebae are found to accumulate in the interlamellar spaces, accompanied by thickening of the secondary lamellae; the lamellae then fuse, with channels and cavities being formed; in advanced infection the normal lamellar structure of the gill filaments disappears completely.

Affected areas of the gill have large numbers of actively secreting mucous cells, present on both the surface and deep within the hyperplastic epithelium; in unaffected areas of gill, mucous cells are either absent or few in number (Roubal *et al*, 1989; Munday *et al*, 1990). Large amounts of mucus have also been found between the secondary lamellae of affected fish (Leiro *et al*, 1998). Chloride cells are reduced in affected areas of the gill, but abundant between the lamellae of unaffected gill filaments (Roubal *et al*, 1989; Munday *et al*, 1990). Dyková *et al* (1995) found numerous chloride cells in the initial stages of lamellar thickening. Elevated blood sodium levels have been reported in clinically affected fish, while subclinically affected fish have lower but still abnormal levels (Munday *et al*, 1990).

Neutrophils are observed early in disease, infiltrating the hyperplastic epithelium and the filament connective tissues; later these cells are mainly mononuclear in character (Roubal *et al*, 1989; Munday *et al*, 1990). The presence of mononuclear nodules, possibly lymphoid in nature, along the primary lamellae and in the basal and interlamellar tissues was reported by Munday *et al* (1990), and thought to be evidence of the development of an immune response.

Paramoeba species are only associated with affected areas, usually confined to the gill surface (Kent *et al*, 1988; Roubal *et al*, 1989; Munday *et al*, 1990; Dyková *et al*, 1998c). Kent *et al* (1988) observed that the severity of the hyperplastic lesions was consistent with the density of amoebae. Examination of sequential samples of Atlantic salmon with AGD in Tasmania showed that pathological changes in the gill filaments were only associated with the presence of *Paramoeba* (Roubal *et al*, 1989). Examination of affected turbot gills showed an almost continuous layer of amoebae observed on the surface of the hyperplastic epithelium, with the numbers diminishing in the late stages of infection (Dyková *et al*, 1995). Kent *et al* (1988) reported that amoebae rarely penetrated the epithelium, however ultramicroscopic examination of the amoebae *in situ*, by Roubal *et al* (1989), showed that gill-attached amoebae were invasive, with pseudopodia penetrating between and into the surface of degenerating epithelial cells.

AGD infected fish, but it has been postulated that the damage may be a direct result of mechanical damage, or may perhaps be due to an unidentified parasite excretory factor (Roubal *et al*, 1989). The reasons for infestation of *Paramoeba* on the gills of these fish are poorly understood, although Roubal *et al* (1989) suggests that under certain environmental conditions, such as increased water temperature, high salinity and poor water exchange, the *Paramoeba* species acts as a primary pathogen.

Characterisation of the pathogen

The *Paramoeba* associated with mortalities of sea-cultured salmonids in the USA has been identified as *P. pemaquidensis* based on the presence of characteristic morphology (Kent *et al*, 1988). Studies of the *Paramoeba* sp. that causes mortalities in sea-cultured Atlantic salmon and rainbow trout in Tasmania also indicate a close resemblance to *P. pemaquidensis* (Roubal *et al*, 1989; Munday *et al*, 1990), but the authors have not assigned a species name to the *Paramoeba* associated with this disease. Kent *et al* (1990) examined histological gill sections from infected salmonids from Tasmania and concluded that the pathological changes were identical to those of salmonids in Washington. More recently, Dyková *et al* (1998c) have identified the causative agent of AGD outbreaks in turbot, in Spain, as belonging to the genus *Paramoeba*, with the principal characteristics of *P. pemaquidensis*. The authors have yet to assign a species name to this amoeba, non-morphological criteria still needing to be assessed.

These pathogenic species of *Paramoeba* share a number of key characteristics with *P. pemaquidensis*: all three isolates showed the presence of fine hair-like filaments on the surface of the cells; a similar overall diameter of the cells; similar size of the parasome and nucleus; and all of the organisms have the ability to thrive in culture - all features consistent with *P. pemaquidensis* (Kent *et al*, 1988; Roubal *et al*, 1989; Munday *et al*, 1990; Dyková *et al*, 1998c). The presence of surface hair-like filaments was not observed in cultured *Paramoeba* by those researchers that investigated the ultrastructure of cultured isolates (Kent *et al*, 1988; Roubal *et al*, 1989; Munday *et al*, 1990). The same groups also reported that cultured isolates were smaller than gill attached organisms. Definitive identification of these isolates as *P. pemaquidensis* may rely on the development of non-morphological methods of identification, such as isoenzyme electrophoresis.

An investigation of the *in vitro* growth characteristics of the *Paramoeba* sp. infecting coho salmon in the US, found the optimal temperature for growth was 20°C, the amoebae growing slowly at 5°C and growth was enhanced with increased temperature up to 20°C. Optimal growth occurred at 15 ppt salinity, with little reduction at 20-30 ppt (Kent *et al*, 1988). However, Paniagua *et al* (1998) found that salinity over the range of 10-30 ppt had no effect on the *in vitro* survival of *Paramoeba* sp. infecting turbot, although incubation temperature was found to be significant, with optimal survival occurring at 11°C, and with survival dropping above and below this temperature. The same authors also reported a direct correlation between incubation time and survival, which was thought to be due to low nutrient availability *in vitro*, or accumulation of toxic products in the agar cultures.

Epizootiology

The most outstanding epizootiological feature of AGD caused by *Paramoeba* sp. is related to temperature; in all cases, outbreaks of AGD have been associated with high sea-water temperatures. In Washington and California, AGD has been reported in autumn associated with temperatures of between 9-13°C (Kent *et al*, 1988). In Tasmania the disease is seen during spring, summer and autumn, associated with temperatures between 12-20°C. Amoebae have also been observed on the gills of salmonids in winter when the water temperatures were close to 10°C (winter and spring), without clinical disease (Foster and Percival, 1988; Roubal *et al*, 1989; Munday *et al*, 1990). In Ireland, outbreaks have occurred following periods of unusual and prolonged extreme environmental conditions, associated with some of the highest sea-water temperatures on record, some sites experiencing water temperatures in excess of 20°C (Rodger and McArdle, 1996; Palmer *et al*, 1997). The same authors reported that clinical disease was observed in the temperature range of 12-15°C. In Spain, the disease in turbot has been reported during the period from October to December, associated with water temperatures between 9.1-18.8°C (Dyková *et al*, 1998c; Leiro *et al*, 1998).

Salinity of the sea-water also appears to be an important epizootiological feature of AGD. Munday *et al* (1990) reported that AGD in Tasmania was only observed when salinities approached 35 ppt. In Ireland, the disease first occurred during a period when rainfall was well below average and farms experienced full oceanic salinity for prolonged periods (Rodger and McArdle, 1996). In turbot, the disease has been associated with salinities between 22-30 ppt (Dyková *et al*, 1998; Leiro *et al*, 1998); however *in vitro* studies have shown no significant relationship between salinity and amoebae survival (Paniagua *et al*, 1998). In contrast Kent *et al* (1988) reported that optimal growth *in vitro* occurred at 15 ppt, with little reduction at 20-30 ppt, growth was slow at between 8-10 ppt and absent at 6-7 ppt. Munday *et al* (1993) also reported that freshwater caused observable damage to amoebae *in vitro*, swelling and loss of mobility, when infected gill filaments were placed into freshwater. Interestingly, early attempts in Tasmania to control AGD involved towing susceptible fish to brackish water sites until the disease period was over, and later the routine bathing of affected fish in freshwater to control the disease, suggest a close relationship between salinity and progression of AGD disease (Roubal *et al*, 1989; Munday *et al*, 1990).

Amoebic gill disease in Tasmania and Ireland usually occurs in young fish, smolt, in their first season in the sea (Munday *et al*, 1990; Rodger and McArdle, 1996; Palmer *et al*, 1997). It was suggested that these fish are susceptible to AGD as they have no developed immunity to *Paramoeba*, and that their osmoregulatory capacity is less than that of larger fish (Munday *et al*, 1990). Kent *et al* (1988) reported that in some outbreaks where mortality was low, only small emaciated fish were heavily infected. In turbot mortalities due to AGD were reported in fish greater than 2kg (Dyková *et al*, 1998c), and also in fish simply labelled 'big specimens' (Dyková *et al*, 1995).

Free-living *Paramoeba pemaquidensis* is widely distributed in the Northern Hemisphere (Page, 1973; Cann and Page, 1982). There are no reports of this species in the Southern Hemisphere, although a *Paramoeba* species resembling *P. pemaquidensis* is thought to be the parasite causing AGD in Tasmania. Jones (1988) investigated the number of *Paramoeba* species in selected water column samples taken from pens of Atlantic salmon infected with AGD, and found one

sample with a total of 3.4 parasites per litre. The numbers were much lower than were expected, particularly when infected fish harboured an average of approximately 4.9×10^6 *Paramoeba* on the gills. However, a study undertaken by Martin (1985) found that *P. pemaquidensis* neither grew nor multiplied when maintained in suspension, and growth only occurred when the amoebae were on a solid substrate, which may explain the low numbers of free-living *Paramoeba* spp. in the water column.

Interestingly, both *P. pemaquidensis* (termed *Neoparamoeba pemaquidensis* by the author) and *P. eilhardi* have been observed on the surface of 5 species of macroalgae, from waters off the Isle of Cumbrae, Scotland (Rogerson, 1991). Small numbers of *Paramoeba* were found on sections sea-cage nets in Tasmania by Jones (1988), however no distinction was made between the net and the algae growing on the nets. He surmised that the dirty nets, although not thought to be a reservoir of infection, could enhance the development of AGD by reducing the water flow through the cages.

Native fish are unlikely to be a reservoir of infection in Tasmania. Jones (1988) observed *Paramoeba* species in the gills of a small number of native fish captured in and adjacent to salmon cages in Tasmania. One native species, *Thyrites atun*, showed severe gill damage associated with infection, and most fish had some degree of damage similar to AGD in salmonids. The presence of *Paramoeba* in these fish suggested to the author that this disease was not exclusive to cultured salmonids. He also suggested that infection in native fish might be a consequence of infected cultured salmonids in the area. However, the presence of heavy infection in barracouta suggested to the author that AGD might be widespread, as barracouta are active open sea predators that are unlikely to confine their activities to the small area of a fish farm.

Unlike mass mortalities of sea urchins due to *Paramoeba*, mass mortalities due to AGD seem to be a phenomenon of aquaculture where large numbers of fish are confined in tanks or sea-cages.

Diagnosis

Initial diagnosis of AGD in salmonids and turbot is by the presence of excessive mucus or mucous patches on the gills (Munday *et al*, 1990, 1993; Dyková *et al*, 1998), or in the case of turbot, clubbed and/or shortened necrotic gill filaments (Dyková *et al*, 1998c). Wet mounts of gill tissue or scrapings from the gills examined by light microscopy, phase contrast microscopy or Nomarski interference phase microscopy, reveal the presence of amoebae with characteristic digitate pseudopodia (Kent *et al*, 1988; Munday *et al*, 1993). The amoebae can be identified as *Paramoeba* sp. by examining locomotive forms, allowed to adhere to glass, by Nomarski interference phase microscopy, and looking for the characteristic parasome (Kent *et al*, 1988). Definitive identification of a *Paramoeba* sp. involved in AGD is usually by examination of haematoxylin and eosin stained gill tissue, looking for amoebae with the characteristic parasome associated with gill damage (Kent *et al*, 1988; Munday *et al*, 1988, 1993; Dyková *et al*, 1998c).

Isolation

Kent *et al* (1988) isolated *Paramoeba* sp. from AGD infected fish by placing the gill tissue into tissue culture medium; Medium 199 (Sigma) containing sea-water, foetal calf serum, chicken serum and various antimicrobial compounds. Isolates were passaged 8 times and then transferred to malt-yeast-seawater agar (as described by Page, 1983) plates seeded with a *Klebsiella* sp., for long term maintenance. All cultures were incubated at 15°C.

The *Paramoeba* sp. affecting salmonids in Tasmania was reported to grow well on malt-yeast-seawater (MYS) agar seeded with *Pseudomonas*, *Klebsiella*, *Escherichia* or *Vibrio* spp, but has not been grown in liquid culture (Munday *et al*, 1990). Roubal *et al* (1989) reported routine maintenance of cultures using MYS agar seeded with *Pseudomonas maltophilia* (now known as *Stenotrophomonas maltophilia*), subculturing every 3-4 weeks by cutting small pieces of agar containing amoebae and placing onto freshly seeded plates. Cultures were incubated at 20°C. There are no accounts in the published literature of primary isolation of this amoeba from the gills.

The *Paramoeba* sp. infecting turbot gills was isolated from gill tissue placed onto cerophyl-seawater (CS) agar and MYS agar (both from Page, 1983), however the growth was reported to be slow and suppressed by the overgrowth of accompanying bacterial flora (Dyková *et al*, 1998c). The authors reported successful growth in liquid culture using CS or MYS medium (Page, 1983), however growth was reported to be slow. Both CS and MYS agars, seeded with a *Pseudomonas* (*Stenotrophomonas*) sp. were used for subcultivation of strains and clones.

Pathogenicity

Most of the evidence suggesting a pathogenic role for *Paramoeba* species in AGD of sea-cage reared salmonids has come from histopathological studies of infected gill tissue, where large numbers of amoebae are observed associated with gill damage (Kent *et al*, 1988; Roubal *et al*, 1989; Munday *et al*, 1990). Roubal *et al* (1989) reported that the amoebae were invasive into and between the surface epithelial cells, a characteristic not observed by Kent *et al* (1988). The presence of this organism in wet mounts of damaged tissue and the ability to culture this organism from infected gills also supports a pathogenic association. Examination of sequential samples of Tasmanian salmon gills over a disease period showed that pathological changes in the gill were only associated with the presence of *Paramoeba* (Roubal *et al*, 1989). Jones (1988) observed that the number of *Paramoeba* in the gills positively correlated with the degree of gill damage, and mortality. In turbot, branchial lesions were never observed without amoebae attached, supporting the pathogenic association of this amoeba (Dyková *et al*, 1995).

Attempts to infect naïve coho salmon in the US and naïve rainbow trout in Tasmania, with cultured *Paramoeba* spp. were unsuccessful (Kent *et al*, 1988; Jones, 1988; L. Searle, Pers. Comm.). However naïve rainbow trout were infected when placed into tanks with infected fish (L. Searle, Pers. Comm.). In the transmission studies undertaken by Kent *et al* (1988) naïve coho salmon were exposed to *Paramoeba* in a bath for 45 minutes, and the fish maintained in a flow through aquarium system. *Paramoeba* were cultured from the tank detritus and all gill samples, up to 29 days post exposure; however no *Paramoeba* were observed in wet gill mounts or in

histological sections. These results indicated to the authors that the cultured *Paramoeba* were not pathogenic under the conditions employed, and that they may only be able to proliferate under certain conditions, such as when there are pre-existing lesions due to other parasites, chemical or mechanical damage. It was also suggested that repeated passage of the cultured amoebae might have reduced their virulence.

Jones (1988) employed a number of innovative methods to infect naïve rainbow trout with cultured *Paramoeba*. Gills were directly flushed with a suspension of *Paramoeba*; fish were exposed to *Paramoeba* in baths containing between 3000-5600 amoebae per ml, for periods ranging of 2-3 hours; gill damage was induced by bathing fish in 2 ppm of benzalkonium chloride; mechanical damage was induced by tying gill arches together with cotton or jute twine; and stress induced by restricting feed and increasing the water temperature. Experimental infections failed, although *Paramoeba* were observed colonising the cotton and jute threads, and were isolated from the tank detritus of one experiment. It should be noted that the numbers of fish in these experiments were small (2-7 fish), and most died early in the experiments.

There is no evidence of any other potential pathogen, such as bacterial, fungal or viral infection, associated with AGD in Tasmania (Roubal *et al*, 1989; J. Carson, Pers. Comm.). However Jones (1988) implied that bacteria may be important in the pathogenesis of AGD, as it is in other cases of branchial amoebiasis where bacteria on the gills are considered a food source for the amoebae (Chatton 1909, 1910; Kubota and Kamata, 1971; Sawyer *et al*; 1975; Nash *et al*, 1988). Dyková *et al* (1995) found no evidence of 'other agents' involved in the tissue changes associated with AGD in turbot, and believed that the distinct foci of the lesions appeared to rule out the negative influences of water quality, as damage would be more diffuse.

Control

Routine control of AGD is only performed in Tasmania, where affected fish are bathed in freshwater, for a period of 1 to 8 hours, or towed to areas of brackish water (Munday *et al*, 1990). In the US the disease occurs sporadically, and is not treated. However Kent *et al* (1988) reported the eradication of disease in fish held in tanks in California following a brief reduction in salinity. It is postulated that freshwater bathing may work in a number of ways: reducing the number of *Paramoeba*; the action of freshwater stripping the excess mucus off the gills, thereby improving oxygen transfer; or by reducing hypernatraemia, at least temporarily, to improve osmoregulation (Jones, 1988; Munday *et al*, 1990). Munday *et al* (1993) reported that freshwater caused observable damage to amoebae *in vitro*, including swelling and loss of mobility, when infected gill filaments were placed into freshwater. Munday *et al* (1990) reported that the levels of *Paramoeba* on the gills after bathing do not reach pre-treatment levels, however, most farms report at least three major waves of infection each year (J. Smith, Pers. Comm.), indicating that the numbers of *Paramoeba* return to levels that precipitate disease.

Bath treatments with formalin, potassium permanganate, chelated copper, malachite green and chloramine T have all been ineffective in treating AGD in Tasmania (Munday *et al*, 1993). In Ireland, freshwater bathing has not been deemed practical due to the location of the various sites. Formalin baths have been tried on two sites, one experiencing a reduction in mortalities due to AGD, and another which continued to experience losses (Rodger and McArdle, 1996; Palmer *et al*, 1997). *In vitro* studies of the *Paramoeba* sp. causing AGD in turbot, in Spain, showed salinities over 10-30 ppt had no effect on *in-vitro* survival, suggesting that freshwater bathing may not be effective in these fish (Paniagua *et al*, 1998). A formalin bath has also been attempted in infected turbot, but was not effective in controlling the course of infection (Dyková *et al*, 1998c).

Despite the ability to control this disease in Tasmania with freshwater, AGD has emerged as a major disease problem, capable of severely threatening the viability of the sea-caged salmonid industry. Amoebic gill disease is the primary disease of concern for the Tasmanian salmonid industry, as identified by regular industry surveys (King, 1994a, 1995). The major reason of this concern is the cost of freshwater bathing and of monitoring to prevent and predict disease; both are labour intensive and may require the purchase of freshwater. The time taken to treat fish is also a limiting factor as only a few pens can be treated at one time, hence farms are under the constant threat of a major outbreak of AGD where they will be unable to treat all pens in time to prevent significant losses (H. King, Pers. Comm.). The level of AGD infection is also an important factor in the timing of freshwater baths, as high mortalities can result when bathing severely infected fish. High water temperatures are another limiting factor for freshwater bathing. At the time of this study farms bathed fish prophylactically, 6-10 weeks after the fish are placed into the sea, with monitoring and bathing of smolt being an ongoing process during early spring, summer, and autumn (J. Smith Pers. Comm.).

1.4 Objectives for this study

Amoebic gill disease (AGD) is an economically significant disease for the relatively young Tasmanian salmonid industry. Despite the success of freshwater bathing, this form of treatment was considered too costly and with a considerable margin of error. The presence of AGD in Tasmania is a limiting factor in scaling up the number of Atlantic salmon able to be successfully reared in the sea. Hence AGD research was made a priority by Salmon Enterprises of Tasmania (Saltas) and its industry members. The objectives for this study were developed in association with Saltas and the salmon farming industry, over the study period.

1. At the commencement of this study there were no surviving isolates of *Paramoeba*. A number of surviving amoebae isolates, cultured from fish with AGD, were in existence, but these amoebae belonged to other marine amoebae genera and were not *Paramoeba* sp. The role of these marine amoebae in AGD is not known. The objectives of this portion of work were to isolate and characterise amoebae from fish with AGD and to determine the pathogenic role of the cultured amoebae, by their association with gill damage in fish with AGD. Once the relationship of the isolates to the disease was understood it would be possible with certainty to: undertake pathogenicity trials; determine the inhibitory effects of proposed treatments; and develop rapid sensitive assays to detect amoeba in clinical material.
2. There is good evidence to suggest that *Paramoeba* is the aetiological agent of AGD, based on histopathological studies where *Paramoeba* can be observed closely associated with gill damage. Attempts to infect naïve fish with cultured *Paramoeba* have been unsuccessful; however naïve rainbow trout exposed to naturally infected fish have developed typical AGD. Hence, there was a need to verify the descriptive reports of pathogenicity ascribed to *Paramoeba* species. The development of a model of infection would provide a means of carrying out, irrespective of season, controlled studies of factors affecting pathogenicity, and allow tank trials of treatments for AGD.

The objectives for this portion of the work were to fulfill Koch's postulates, by exposing naïve fish to the authenticated amoeba isolate to precipitate AGD; and to verify and optimise the infection of naïve fish using AGD infected fish.

3. While freshwater bathing is successful there may be other more effective and economic means based on medicated baths or feeds. Field trials of potential chemotherapeutants and treatments are costly and time consuming, and therefore not effective for large-scale screening. The development of an *in vitro* method would provide a rapid and cost effective means to screen potential chemotherapeutants and treatments. In addition, the action of freshwater in controlling AGD is not fully understood, it has been suggested that freshwater may be amobicial, although this has not been proved.

The objectives of this work were as follows; develop a method of screening potential amoebicides for their effect on the authenticated amoeba *in vitro*; identify and screen potential amoebicides; and to determine the effect of freshwater on the amoeba *in vitro*.

The results of such a study would determine which amoebicides would be tested in the field, and provide evidence for the therapeutic effect of freshwater.

4. On the farms the process of avoiding outbreaks of severe AGD, and hence the devastating losses that could ensue, requires constant monitoring and bathing of individual fish cages, which is both costly and time consuming. The gills of fish are examined for mucous patches, however the margin for error is high, as fish can rapidly succumb to disease by the time mucous patches are observed. Therefore, the objective of this part of the study was to develop a sensitive diagnostic assay, allowing early prediction of AGD in fish; and to investigate any other means of predicting outbreaks or improving the diagnosis of AGD.

5. Amoebic gill disease in Tasmania occurs primarily in smolt, during their first year in the sea. Serious outbreaks have not been reported in larger, harvest size fish, during their second year, leading researchers to suggest that some form of immunity is acquired. The objective here was to develop an assay to detect the presence of the antibodies to the authenticated amoeba species in serum and gill mucus, and to investigate the presence of specific serum and/or mucus antibody in salmon naturally exposed to AGD in the field.

Chapter 2. Methods

INTRODUCTION

Despite a number of studies in Tasmania (Roubal *et al*, 1989; Jones, 1988; Munday *et al*, 1990), at the commencement of this study there were no authentic strains of *Paramoeba* sp. 'AGD' available. There were however a number of amoeba isolates collected from the gills of fish with AGD that proved on closer examination not to be *Paramoeba* spp. Cultures of *Paramoeba pemaquidensis* (Kent *et al*, 1988) were also unable to be retrieved from cryopreserved samples lodged with the American Type Culture Collection (B. Robinson, Pers. Comm.). This evidence suggested that *Paramoeba* sp. 'AGD' were difficult to both isolate and maintain in long term culture.

The factors affecting the isolation of *Paramoeba* sp. 'AGD' from the gills of Atlantic salmon, and the maintenance of *Paramoeba* sp. 'AGD' as *in vitro* cultures were largely undefined. These had to be determined empirically during the course of this work, adapting a variety of methods used in the study of marine and freshwater amoebae, including other species of *Paramoeba*, as well as common methods used in the study of bacteria.

The individual studies discussed in this thesis were not conducted in a chronological order, most being conducted concurrently, as is often the case with commercial based research, leading to methods being developed during the course of this work. The methods presented in this chapter represent the standard methods used in this study, the development of which will be discussed in the relevant chapters. In some cases other methods were employed that were later superseded by the methods in this chapter, these will be discussed in the relevant chapters. The methods presented in this chapter have been described in detail due in large part to the difficulties observed during the present study in the culture, maintenance and identification of *Paramoeba* sp. 'AGD', as well as the inherent problems encountered when adapting this organism to methods established for the study of other micro-organisms. They are intended to serve as a standard methodology for future studies of amoebic gill disease of salmonids in Tasmania.

2.1 Culture of marine amoebae

The following methods describe the techniques used for cultivation of *Paramoeba* sp. 'AGD', and other marine amoebae isolated from the gills of sea-cage farmed Atlantic salmon, based on the methods described by Page (1983). Euryhaline organisms (such as *Acanthamoeba* spp.) were often isolated from the marine environment; the techniques for their cultivation are essentially the same but substituting sea-water for freshwater. Agar plate cultures were utilised for long term culture maintenance, and growing large numbers of amoebae required for antigen or infection trials. Attempts in the present study to grow *Paramoeba* sp. 'AGD' in liquid culture have not been successful.

Choice of agar

Malt yeast agar (Appendix 1.3) was the preferred agar for isolation and maintenance of amoeba cultures, although sea-water agar (Appendix 1.5) was also used.

Freshwater agar (Appendix 1.4) was used primarily for the isolation of cyst forming euryhaline and freshwater amoebae. In some cases a 'cocktail' of antibiotics were added to the agar at a ratio of 1:1000 to reduce the overgrowth of normal bacterial flora (Appendices 3.1 and 3.2)

Apparatus

A dissecting microscope with adjustable sub-stage lighting and glass stage, magnifying to at least X50, was used for cloning and subculture of amoebae. To reduce fungal contamination, manipulation of cultures was performed in a Class II Biological Safety Cabinet.

2.1.1 Seeding agar plates

Bacterial growth substrate: Initially all marine amoebae were isolated and maintained on a bacterial culture of *Flexibacter maritimus* (Appendix 2.2) but was this changed to *Stenotrophomonas maltophilia* (Appendix 2.1), as discussed in Chapter 3. *E. coli* (Appendix 2.1) was the preferred bacterial substrate for *Acanthamoeba* species. Marine amoebae could also be cultured on marine bacteria isolated from gill tissue (Appendix 2.3); this mixed marine bacterial substrate was used in the culture of *Paramoeba* sp. ‘AGD’ for use in pathogenicity trials (Chapter 4).

Standard 90mm petri dishes: These were used for routine culture and maintenance. Plates were seeded with between 100 and 150 µl of the appropriate bacterial suspension, the volume dependent on the dryness of the plates. A sterile disposable plastic spreader (Oxoid) was used to distribute the bacterial suspension over the surface of each plate.

Large 145mm petri dishes: Nunc plates (Cat # 240401) were used to scale up the cultures of *Paramoeba*. Between 750µl and 1.0ml of bacterial suspension was used to seed each plate.

Nunc 245mm² Bioassay plates: Nunc bioassay plates (Cat # 240835) were used in large scale production of amoebae. Between 3 and 5ml of bacterial suspension was used per plate.

2.1.2 Subculture

Plates were routinely subcultured as follows: A suitable group of amoebae was located from the growing front of the culture using a dissecting microscope. Using a sterile scalpel blade a square of agar containing the amoebae was cut, removed and inverted onto a newly seeded agar plate. For routine maintenance of the isolate, the block was placed close to the edge of the plate, allowing the width of the plate for the

amoebae to migrate. For harvesting or large-scale production, a new plate was inoculated in the centre to allow symmetrical growth. The plates were inverted and sealed in a plastic container or bag, and incubated at 20°C. *Paramoeba* sp. 'AGD' are heat labile, and cultures were never exposed at any time to temperature in excess of 22°C. Cultures were checked weekly to ensure growth. Old cultures were retained for at least 1 week.

Plates were subcultured once the bacterial substrate had been consumed, seen as a clearing of the bacterial substrate. Subculture rates varied between different isolates of *Paramoeba* sp. 'AGD', but typically subcultures were made every 3-4 weeks.

2.1.3 Large-scale production

Larger numbers of amoebae for antigen preparation, infection trials or drug tests were grown by a step-wise scaling up of agar plate cultures using 145mm petri dishes and Nunc Bioassay dishes.

Amoebae were harvested from a 90mm plate by flooding the plate with 3-5ml of sterile sea-water [SSW] (Appendix 1.1), and gently dislodging the amoebae from the surface of the agar using a sterile spreader. The harvest was collected using a sterile Pasteur pipette and spread across the surface of a freshly seeded 145mm diameter petri dish, using a sterile spreader. The new plates were then incubated at 20°C for 7-10 days.

Once the bacterial substrate appeared exhausted, harvesting was repeated, using 5-8ml of SSW, splitting the amoeba suspension between three 145mm petri dishes or two bioassay dishes. Further scale up was achieved by repeating the harvesting and splitting cultures.

2.1.4 Contamination control

Contamination of cultures by bacteria, motile protozoa and particularly fungi occurred from time to time, and was time consuming to eradicate. To avoid contamination, the following steps were observed: strict aseptic techniques were employed during all manipulations of amoebae; a Class II Biosafety Cabinet was used for all seeding, subculturing and harvesting; purity checks were routinely performed on the bacterial substrate; sea-water and reverse osmosis (R.O.) water was autoclaved before use (Appendices 1.1 and 1.2).

To keep cultures free from fungal contamination pimaricin (Sigma, P0440) a broad spectrum fungicide (Pedersen, 1992) at 12.5µg/ml was added to the agar. In the event of fungal contamination subculture was undertaken more frequently, and fresh cultures made by collecting amoebae from the point furthest from the fungal contamination.

Contamination by bacteria other than the bacterial growth substrate was observed by the occurrence of individual bacterial colonies dissimilar to the substrate bacterium. Highly motile protozoa (not identified) were sometimes encountered, seen as fast moving bodies often with visible flagella under the light microscope. To avoid such contamination, the plates were kept relatively dry. In the event of contamination a range of antibiotics (Appendices 3.1 and 3.2) was added to agar plates at a ratio of 1:1000 and fresh cultures made by collecting the amoebae at a point furthest away from the contaminated area, several subcultures were usually required to completely eliminate a contaminant. Antibiotics were used by Kent *et al* (1988) and not found to inhibit *Paramoeba* growth or survival, and were not found to effect the survival of marine amoebae in this study.

2.1.5 Revival of failing cultures

Paramoeba sp. 'AGD' are relatively slow growing and especially susceptible to high temperatures; poor growth was observed on plates that were too dry. Apparently dead or dying cultures could often be revived by the addition of extra bacterial substrate direct to the plates and keeping them relatively wet. After several days the remaining amoebae were harvested and inoculated onto a fresh plate.

2.2 Isolation of amoebae from fish gills

Several methods were used for isolating marine amoebae from the gills of AGD infected fish (Chapter 3), but only the ammonium chloride wash could recover adequate numbers of *Paramoeba* sp. 'AGD'.

2.2.1 Ammonium chloride wash

This method was used to isolate marine amoebae, particularly *Paramoeba* sp. 'AGD', and some euryhaline amoebae, from gill tissue. It is a modification of the method described by Jones (1988).

For optimum isolation of *Paramoeba* sp. 'AGD', fish with advanced AGD (greater than 10 mucous patches) were selected. Fish were euthanased by overdose with benzocaine (70-100mg/L in sea-water). Once dead, the head was removed, to stop excess blood from covering the gills, and the gill arches excised. Gill arches were placed into 50ml plastic centrifuge tubes containing enough ammonium chloride solution (Appendix 3.3) to cover the tissue, and shaken for 1-2 hours on a shaking table at 20°C. Samples taken in the field, where no laboratory facilities existed, were transported back to the laboratory in an insulated container prior to processing. After washing, the gills were removed and the freed cells concentrated by centrifugation (1000g for 15-20 minutes). The supernatant was then removed and the pellet of cells resuspended in approximately 40ml of SSW (Appendix 1.1). The cells were then centrifuged again, the supernatant removed and the pellet resuspended in 1-5ml of

SSW (= gill wash material). Approximately 250-500µl of gill wash material was inoculated onto 90mm malt yeast agar plates freshly seeded with *S. maltophilia* (Appendix 2.1) and spread across the surface of the plate with a sterile spreader. Between 3-6 plates were prepared for each set of gills; or spread onto large 145mm petri dishes or 245mm² bioassay plates, to allow separation of amoebae. Plates were incubated at 20°C and observed every two days, for a total of two weeks for plaques indicated by clearing of the substrate by the amoebae. Isolates were cloned as described below (Method 2.2.2).

2.2.2 Cloning of amoebae

To obtain pure cultures, the amoebae were cloned from single cells taken from plaques located on a primary isolation plate (Method 2.2.1). Plaques of amoebae were located using a dissecting microscope, and marked with a permanent marker. A freshly seeded MYA plate (Appendix 1.3) or FWA (Appendix 1.4) plate for freshwater and/or euryhaline amoeba, was marked with 4-6 evenly spaced dots, using a permanent marker. Small numbers of amoebae were located within the plaque and a small volume of SSW (5-10µl) added to the cells and the cells removed using a drawn out Pasteur pipette. If a single cell was removed, then the cell was expelled from the pipette onto one of the pre-marked dots on fresh plate. In cases where more than one cell was removed the amoebae were expelled onto an unseeded MYA plate and additional SSW spread over the surface using a sterile spreader to separate the cells allowing single cells to be removed as described. The cultures were incubated at 20°C and the clones checked for growth every two days. Once the single cells had multiplied to approximately 50 cells they were subcultured as described in Method 2.1.2.

Purification of cyst forming, euryhaline amoebae was achieved in essentially the same way except that only the trophozoite (locomotive form) was selected for purification and cloning, and not the cysts (seen as highly refractive, grain-like structures).

2.3 Identification of *Paramoeba* species

The methods described here are confined mainly to identification of the parasome, a complex DNA containing body adjacent to the nucleus, a distinguishing feature of the genus *Paramoeba*. The parasome could be identified by phase contrast microscopy (Method 2.3.1) and specific DNA staining either through the use of DAPI (Method 2.3.2) or Gomori's trichrome stains (Method 2.3.3). Three methods were investigated to allow identification of the parasome in both the laboratory and the field (as discussed in Chapter 3). Phase contrast microscopy was also used to identify other morphological characteristics of marine amoebae species, as discussed in Chapter 3.

2.3.1 Phase contrast microscopy

This method was adapted from the method described by Page (1983). Amoebae were removed from the growing front of a pure culture by dispensing approximately 100µl of SSW (Appendix 1.1) onto the front and removing amoebae by aspiration in a Pasteur pipette. The amoebae were placed onto a microscope slide coated with agar (Appendix 4.1) so that the cells would adhere to the agar and assume a locomotive form. The flattened amoebae could then be observed for the presence of one or parasomes, adjacent to the nucleus (see Figure 3.6, Chapter 3). To observe the floating form, the aspirated amoebae were placed onto an uncoated microscope slide. Amoebae were observed for morphological characteristics, as described in Table 3.7a & 3.7b (Chapter 3), at between 100X and 1000X magnification on a light microscope with phase contrast objectives. These tables were compiled to describe the common features of the amoebae genera isolated from gills, using selected observations made during the characterisation of amoebae from fish with AGD (see Chapter 3).

2.3.2 DAPI-staining

This staining method provides a rapid alternative to other staining methods or electron microscopy, for the unequivocal demonstration of parasomes. DAPI (4'-diamidino-2-phenylindole) is specific for staining chromatin, found in nuclei and parasomes (Rogerson, 1988).

Cells were harvested from actively growing pure cultures with 5ml of SSW and fixed with formalin (37% formaldehyde), to give a final concentration of 3% v/v. To each 1.0ml aliquot of cells, 50µl of DAPI (Appendix 4.2) solution was added, and incubated for 15-30 minutes in the dark at room temperature. The suspension was filtered onto a 0.6µm black polycarbonate membrane (Sartorius, Cat No. 13005) and partially dried by passing 2-5ml of air through the filter. The filter was mounted on a microscope slide using a non-fluorescing immersion oil. The filter was examined at 200-400X magnification using a Leitz Dialux microscope with epifluorescence (Objective: Leitz Plan Achromat Series, Filter Pack A), to identify amoebae with highly fluorescent nuclei and parasomes. *Paramoeba* spp. were identified by the presence of more than one fluorescent body per cell.

2.3.3 Gomori's trichrome stain

This stain is specific for chromatin (Spencer and Monroe, 1961). *Paramoeba* species were identified by the presence of more than one stained body: the nucleus and one or more parasomes.

Amoebae were harvested from actively growing plates with 2-3ml of SSW, and 1-2 drops of the suspension placed onto one end of a chrom gelatin coated slide (Appendix 4.1), and the slide tilted to allow the drop to run to the other end. The slides were fixed immediately in Schaudinn's solution (Appendix 4.4), for 30 minutes. Slides were immersed in 70% alcohol for 5 minutes; and then for 3 minutes in 70% alcohol with sufficient iodine crystals to produce a port wine colour; and finally in 2 changes of 70% alcohol, for 1 minute each. The slides were then

immersed in Gomori's trichrome stain (Appendix 4.5) for 8-15 minutes and rinsed in 90% alcohol with 1% acetic acid for 1-2 seconds, dipped twice in 100% alcohol, and finally dehydrated in a second change of 100% alcohol for 30 seconds. Slides were cleared in X₃B for 1 minute, and mounted with Permount (Fisher Scientific, SP15-500). After mounting, the slides were examined for red/purple stained nuclei and parasomes.

2.4 Cryopreservation of amoebae

2.4.1 Cryopreservation

The general procedure is based on a method to cryopreserve freshwater amoebae as described by Robinson *et al* (1990).

Pure cultures of amoebae were harvested from actively growing plate cultures by flooding the plate with SSW (Appendix 1.1) for marine amoebae, sterile R.O. water (Appendix 1.2) for freshwater amoebae, or 50% SSW/sterile R.O. water for euryhaline species, and dislodging the cells from the surface using a sterile spreader. Freshwater and euryhaline amoebae were allowed to encyst prior to cryopreservation. The amoebae were concentrated to 1.0ml by centrifugation for 20 minutes at 1000g, and finally pelleted in a microfuge for 1 minute at 12,000g. After pelleting, the amoebae were resuspended in 1.0ml of freshwater cryopreservation medium (Appendix 9.1) for freshwater and euryhaline amoebae or marine cryopreservation medium (Appendix 9.2) for marine amoebae, and mixed thoroughly by vortexing. The cells were incubated in the cryopreservation media for at least thirty minutes, at room temperature, to allow the dimethyl sulphoxide (DMSO) to enter the cells. Cells were then aliquoted into 250µl volumes into 1ml cryotubes (Nunc, Cat No. 375353). The cells were then placed at 4°C for 1 hour, and then directly in an ultra deep freeze at -70°C.

2.4.2 Culture retrieval

Amoebae were removed from -70°C and thawed rapidly by placing the vial in a 37°C water bath. The frozen culture was observed continuously during thawing and removed immediately upon thawing. The amoebae were allowed to stand at room temperature for at least 1 hour. The cells were pelleted by centrifugation at 10,000g for 1 minute, to remove the cryopreservative, and the pellet resuspended in 1.0ml of SSW, 50% SSW or R.O. water as appropriate. The appropriate plates were then inoculated with drops of cell suspension. For marine amoebae malt yeast agar plates (Appendix 1.3) were used, freshly seeded with *S. maltophilia* (Appendix 2.1). For freshwater or euryhaline amoebae, freshly seeded freshwater agar plates (Appendix 1.4) were each inoculated with 0.5ml of cell suspension, and the plates kept moist with sterile R.O. or 50% SSW as appropriate. The plates were then sealed in plastic bags and incubated at 20°C, and examined every two days for growth. The marine amoebae were subcultured upon evidence of cell migration away from the point of inoculation. Freshwater and euryhaline amoebae were subcultured once the amoebae had excysted by removing the trophozoites and inoculating fresh plates.

2.5 Antigen preparation and antiserum production

2.5.1 Antigen preparation

Actively growing amoebae were harvested from a large culture plate (Method 2.1.3) by flooding the plate with SSW. Harvesting was repeated 3 times to ensure that all the amoebae were removed from the plate. The amoebae were concentrated at room temperature by centrifugation at 1,000g for 20 minutes. The supernatant was discarded and the pellet washed in SSW and centrifuged again, washing being repeated at least three times to remove as much of the bacterial substrate as possible. After washing, the pellet was resuspended in 1-3ml of SSW and the total number estimated using a haemocytometer (Appendix 5). The cells were then lysed by sonication using a Branson sonifier fitted with a microtip. Disruption was achieved using 3 pulses of 10 seconds each, output setting 7, equivalent to 60 watts. The tubes

were sonicated on ice to prevent heating of the antigen. After sonication the cell debris was centrifuged at 2,000g for 20 minutes. The supernatant was removed and any live bacteria remaining inactivated by the addition of formalin to achieve a final concentration of 0.5% v/v. The antigen was refrigerated overnight before inoculation into animals. If not used within 24 hours the antigen was aliquoted and stored at -20°C.

For inoculation into fish or antigen for ELISA microplate coating, the protein concentration of the antigen was determined using the BIORAD Protein Assay Kit (Cat # 500-0001). Antigen for ELISA's was prepared as described, without the addition of formalin.

2.5.2 Antiserum production in rabbits

The rabbits used in this study were bred and maintained in a specialised animal facility located at the Animal Health Laboratory, DPIWE, Launceston, Tasmania.

Antigen was injected into the marginal ear vein of the rabbit using a 25 gauge needle. An initial dose of 0.1ml was administered, followed at 3 day intervals with increasing volumes of 0.2ml, 0.4ml, 0.6ml, 0.8ml and finally a 1.0ml dose. Rabbits were monitored closely for signs of anaphylactic shock, which was occasionally observed, these animals were euthanased by a veterinarian.

A test bleed was taken 14 days after the last injection and the titre checked by an immunofluorescence assay (Method 2.6.3). Titres $\geq 1:100$ were considered useful for immunological procedures. In rare cases where the titre was low, a booster inoculation of 1.0ml was given. Blood was separated as described in method 2.5.4.

In most cases the number of amoebae injected over the period to produce an acceptable antibody response was from approximately 100,000 – 400,000 for dose 1 to approximately 1-4 million amoebae for dose 6.

2.5.3 Anti-serum production in fish

Naïve freshwater rainbow trout were used for anti-serum production, marine fish were not used due to possible exposure to *Paramoeba* sp. 'AGD'. The fish ranged from 60-80g in weight. This method was modified from the method published by Bryant *et al* (1995). Rainbow trout were maintained in a specialised freshwater tank facility located at the Animal Health Laboratory, DPIWE, Launceston, Tasmania, as described in Chapter 7.

Paramoeba sp. 'AGD' antigen was prepared, as described in Method 2.5.1. The protein concentration of the antigen was determined, and the concentration adjusted to 1mg/ml representing approximately 5,000,000 *Paramoeba* per ml. The antigen was further diluted in SSW to give protein concentrations of 25µg, 50µg and 100µg per 0.1ml, and emulsified with an equal volume of Freud's complete adjuvant (CSL), to give an injectable dose of 0.2ml.

Fish were anaesthetised in benzocaine (45mg/L) and placed on a wet paper towel to keep them moist. Antigen was inoculated by intraperitoneal injection slightly lateral to the mid ventral line, halfway between the pelvic and pectoral fins, using a 23 gauge needle. Fish were maintained in freshwater at 15°C, in a flow through tank system (as described in Chapter 7) and re-immunised 4 weeks after the initial dose. Fish were bled 2-4 weeks after the second dose. Blood was separated as described in Method 2.5.4.

Bleeding Fish: When fish were needed to be kept alive they were anaesthetised in benzocaine (45mg/l), fish required for terminal bleeds were euthanased by overdose with benzocaine (70-100mg/l). Blood was drawn from the caudal vein, located midway between the anus and the tail, using a 21 gauge needle (Clarke, 1990). The volume collected from each fish varied depending on the size of the fish and the need to keep it alive, approximately 2ml for small fish (100g-500g) and up to 10ml from larger fish.

2.5.4 Serum preparation

After collection, blood was allowed to clot for 30-60 minutes: 37°C for rabbit blood and 20°C for fish blood. After clotting, a Pasteur pipette was used to separate the clot from the sides of the vessel and the sample held at 4°C overnight. After refrigeration the serum was removed from the clot using a sterile pipette, and the remaining serum separated by centrifugation at 2,000g for 15 minutes. Serum was aliquoted and stored at -20°C. Rabbit serum used in the IFAT (Method 2.6.3) was diluted 1:2 in 100% glycerol and some retained as a reference without glycerol.

2.6 Gill health assessment methods

Gill health in fish infected or at risk of infection with amoebic gill disease was assessed using three methods:

1. Gross examination
2. Histological examination
3. AGD Immunofluorescence antibody test (IFAT)

The choice of methods used to assess the level of infection varied according to the information required. Gross examination of the gills of the fish was undertaken if the fish studied were not to be sacrificed, or to provide a quantitation of the gross damage in sampled fish. To determine damage at the cellular level histological examination was performed. This method was able to determine the extent of the damage to the gills, and to differentiate between AGD and other gill health associated pathologies. The AGD IFAT was developed to reliably identify the presence of *Paramoeba* sp. 'AGD' in smears from gill tissue. It is now routinely used to diagnose amoebic gill disease in cages of Atlantic salmon.

2.6.1 Gross examination

Fish were anaesthetised in benzocaine (45mg/l) and gills exposed by placing a forefinger into the mouth of the fish and pushing the gills outwards. Gills were examined for the presence of mucus and mucous patches, observed as white patches varying in size according to the size of the fish and severity of infection (Alexander, 1991). The number of patches present on the gills were scored using the following scale:

-	No mucus, no patches
±	Little mucus, no distinct patches
+	1-3 patches
++	4-6 patches
+++	7-9 patches
++++	> 10 patches

2.6.2 Preparation of gill tissue for histological examination

Fish were euthanased by overdose with benzocaine (70-100mg/l). Immediately following death, the head removed to prevent contamination of the gills with blood. The operculum was removed to expose the gills, two gill arches excised from each side of the fish, and placed immediately in the appropriate fixative (Appendices 6.1 – 6.5). The fixatives used varied over the course of this study, their relative merits in AGD diagnosis was investigated in Chapter 6.

Gills were mounted in paraffin wax, 5-10µm thin sections cut and sections stained with haematoxylin and eosin (H&E) using standard histological processes.

Gill sections were examined for the following features, as described by Kent *et al* (1988) and Munday *et al* (1990):

- Hyperplasia or fusion of the secondary lamellae
- The number of mucous and chloride cells
- The presence and density of lymphoid nodules
- Integrity of the lamellar epithelium
- The presence or absence of amoebae

2.6.3 Immunofluorescence antibody test (IFAT)

This method was developed to detect *Paramoeba* sp. ‘AGD’ in gill smears, as described in Chapter 6.

Sample collection

Smears were taken using one of two methods. In live fish, not for sacrifice, samples were taken by anaesthetising the fish in benzocaine (45mg/l) and scraping the area of gill selected using the clean edge of microscope slide or other blunt instrument. The mucus was then smeared across a clean microscope slide, to cover approximately two thirds of the working area of the slide. Slides were then air dried, or dried quickly on slide warmer at 50°C.

Alternatively mucus was collected from dead fish or sacrificed fish by removing a selected gill raker and smearing the gill tissue across a clean microscope slide, covering approximately two thirds of the working area of the slide. Slides were then dried as described.

IFAT test

Smears were fixed by passing the slide face up, through a naked Bunsen flame once or twice. Positive control smears (Appendix 8.1) were included with each assay. The area of the smear was circled using a wax pencil or hydrophobic marking pen, such as the Pap Pen®. Slides were flooded with PBS (Appendix 8.2) and allowed to stand for 3-5 minutes at room temperature. The PBS was tipped off and the moisture around the marked area wiped off. The primary antibody, rabbit anti-*Paramoeba*, diluted 1:100 in IF antibody diluent (Appendix 8.3) was added to the test area, approximately 350µl of diluted antibody was used per smear. Antibody was incubated at 37°C for 60 minutes in a moist chamber. Slides were washed twice in two changes of PBS, for 5 minutes at room temperature. After washing the slides were wiped dry as described. The secondary antibody, fluorescein labeled anti-rabbit IgG (Silenus, Cat No. 984131020), diluted 1:60 in PBS, was added to each smear. Slides were incubated at 37°C for 30-45 minutes in a moist chamber. Slides were washed twice as described, mounted in alkaline buffered glycerol (Appendix 8.4), and examined at X100 magnification with a Leitz Dialux 20 UV microscope and an I2 filter pack. At least 10 fields of view were observed per slide. Tests were considered valid only if the positive control contained brightly fluorescing yellow/green cells (See Figures 6.1 and 6.2, Chapter 6).

Smears were scored as follows:

- +/- occasional (1-2 cells per 10 fields)
- + small numbers (3-10 cells)
- ++ moderate numbers (11-20 cells)
- +++ many (> 20 cells)

2.7 Immunostaining techniques

2.7.1 Immunofluorescence

This method was developed to rapidly screen fixed or frozen gill tissue with anti-sera to selected marine amoebae (see Chapter 3).

Sections, cut approximately 5-10µm thick, were placed onto slides pre-treated with a tissue adhesive, Poly-L-Lysine® (Appendix 7.1). Fixed sections were deparaffinised to remove the wax (Appendix 7.2). Frozen sections required no pre-treatment and were immunostained immediately after cutting. Sections were rinsed briefly in PBS, pH 7.2 (Appendix 8.2) and the anti-amoeba rabbit antibody, diluted 1:100^{a1} in IF antibody diluent (Appendix 8.3), applied to each section and the sections incubated at 37°C for 30-60 minutes, in a moist chamber. Sections were washed in 3 changes of PBS, for 5 minutes each. After washing fluorescein labeled anti-rabbit IgG (Silenus, Cat No. 984131020), diluted 1:60 in PBS, was added to each section, and the sections incubated at 37°C for 30-60 minutes, in a moist chamber. Finally, the sections were washed as described and mounted in alkaline buffered glycerol (Appendix 8.4). Sections were examined under the UV microscope (Leitz Dialux 20 with I2 filter pack), at 100X magnification, for highly fluorescent green/yellow cells (Figures 3.20 to 3.22, Chapter 3).

2.7.2 Immunoperoxidase

This method was developed to detect the presence of *Paramoeba* sp. ‘AGD’ in fixed gill tissue samples already screened positive by immunofluorescence and specifically to visualise the relationship of the cells with the gill tissue (see Chapter 3).

¹ The antibody dilution was determined by titrating the antibody against the cultured amoeba isolate, smeared onto Poly-L-Lysine® coated slides. A dilution of 1:100 was the optimum dilution for all antibodies tested by immunofluorescence, giving the best possible fluorescence without excessive background fluorescence.

Tissues were cut and deparaffinised as described (Method 2.7.1) and then rinsed briefly in PBS. Sections were incubated at 37°C for 30-60 minutes with rabbit anti-*Paramoeba* antibody diluted 1:100 in IP antibody diluent (Appendix 8.5), in a moist chamber. Sections were then washed 3 times in PBS pH 7.2 (Appendix 8.2), 5-10 minutes each wash. Vectastain biotinylated antibody (Vectastain ABC kit, Vector Laboratories, USA, Cat No. PK-4001) was prepared by diluting 1:200 in PBS, and incubated on the sections for 30 minutes at 37°C, as described. After incubation, the sections were washed again, as described above. The sections were then covered with the diluted Vectastain ABC reagent, reagents A and B diluted 1:100 in PBS, and the sections incubated for between 30 and 60 minutes at 37°C, as described. The sections were washed again, as described, then incubated for 2-7 minutes in DAB peroxidase substrate solution (Appendix 8.6) in the dark, and the reaction stopped by washing in running tap water for 5 minutes. The slides were counterstained in haematoxylin (Appendix 8.8), and mounted in DPX mountant. Sections were examined under a light microscope at 200-400X magnification for the presence of reddish/brown amoebae against a pale pink background (Figures 3.23 to 3.25 Chapter 3).

2.7.3 Alkaline phosphatase

This method was used as an alternative to immunoperoxidase staining as it provided a better overall contrast between *Paramoeba* sp. 'AGD' and counterstained gill tissue.

Sections were cut and probed as described for immunoperoxidase staining (Method 2.7.2), using the Vectastain ABC-AP Kit (Vector Laboratories, USA, Cat No AK500). Colour was developed using the Vector blue substrate (Vector Laboratories, USA, Cat No. SK-5300), reagent 1, 2 and 3 diluted 1:100 in AP substrate buffer (Appendix 8.7), for 5 minutes in the dark. The reaction was stopped in gently running water for 5 minutes, and the slides immediately counterstained in haematoxylin (Appendix 8.8). Slides were mounted in DPX mounting medium, and examined under a light microscope at 200-400X magnification for the presence of dark blue stained amoebae against a pale pink background (Figures 3.27 to 3.28, Chapter 3).

2.8. Preparation of *Paramoeba* for electron microscopy

This technique was used to examine the ultrastructure of *Paramoeba*, and in particular for the presence of hair-like filaments on the cell surface (see Chapter 4). Amoebae were fixed and stained using a modification of the method described by Jones (1985).

Actively growing plate cultures of *Paramoeba* sp. 'AGD' were harvested with approximately 5ml of SSW (Appendix 1.1), and concentrated by centrifugation at 1000g for 20 minutes. The supernatant was discarded and the pellet resuspended in approximately 500µl of SSW and fixed in 10 volumes of glutaraldehyde-cacodylate fixative (Appendix 10.2) for 30 minutes. The pellet of cells were rinsed 3 times in sodium cacodylate buffered sea-water (Appendix 10.1), and centrifuged as described. The pellet was transferred to a 1.5ml microfuge tube, concentrated in a microfuge for 30 seconds at 12,000g, and as much of the supernatant removed as possible. A 2% solution of non-nutrient agar was prepared, in R.O. water, and heated in a microwave oven to dissolve the agar. The agar was allowed to cool to 45–50°C, until almost set. The pellet was resuspended in 2-3 drops of the molten agar, and then allowed to cool and set. Once set, the agar block was removed from the microfuge tube and fixed in 1% osmium tetroxide in cacodylate buffer (Appendix 10.3), for 1 hour. The block was then rinsed in distilled water for 5-10 minutes and immersed in 5% uranyl acetate (Appendix 10.4) for 30 minutes to stain the amoebae. After staining, the block was dehydrated in an ascending series of alcohol concentrations, with two changes of 10 minutes duration in 50%, 75% and 95% ethanol. The block was then stored in 95% ethanol prior to processing for electron microscopy.

Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined in a Hitachi H300 transmission electron microscope at 5,000X magnification to observe the structure of the parasome, and 40,000X magnification to observe the surface membrane.

2.9 Chemical screening assays

2.9.1 Preparation of chemicals

Stock solutions containing 3000µg/ml (100X concentrate) were prepared, in 10ml volumes. Chemicals were solubilised as per solubility data provided in The Merck Index solvents (11th edition, 1989). Chemicals solubilised in ethanol were prepared at a concentration of 6000µg/ml, in 5 ml of solvent, as ethanol at higher concentrations in the agar was found to be amoebicidal to *Paramoeba* sp. 'AGD'. Dimethyl formamide and acetone were found to be amoebicidal, and therefore were not used as solvents.

All chemicals were filter sterilised using disposable 0.22µm nitrocellulose filter units. In some cases the solvent was unable to be filter sterilised using nitrocellulose membranes, in which case they were tested without sterilisation. Chemicals unable to be solubilised were tested as suspensions. All stock solutions were covered with aluminum foil to protect light sensitive chemicals, and incorporated into agar preparations within 2 days of preparation.

2.9.2 Growth inhibition assay

This method was used to determine the ability of *Paramoeba* sp. 'AGD' to grow and/or survive in the presence of selected chemicals, and to determine the minimum inhibitory concentration (MIC). The results of which were used to identify chemotherapeutants that could be used in feed to treat fish infected with amoebic gill disease and to select which chemicals were to be tested by the contact inhibition assay (Method 2.9.3).

Agar preparation

Individual volumes of growth inhibition agar (Appendix 1.8) were prepared for each concentration of chemical together with a solvent control. Each volume was sufficient for at least 10 wells of a 5 X 5 ‘Repli-dish’ (Sterilin, UK). Agar was allowed to cool to approximately 60°C before adding the chemicals. Chemicals and solvent controls were diluted in sterile R.O. water (Appendix 1.2) to achieve concentrations of 300, 200, 150, 100 and 50µg/ml from the stock solution (Method 2.9.1). To each individual 22.5ml volume of growth inhibition agar, 2.5ml of each diluted chemical or solvent control was added, and the agar mixed. Two Repli-dishes were prepared for each chemical and solvent control, according to the layout in Figure 2.1. To each well, 1.5ml of agar was added, containing the appropriate chemical or solvent dilution. Negative control plates were prepared by adding 1.5ml of growth inhibition agar, with no chemical or solvent, to the wells of Repli-dishes. Plates were dried in a sterile laminar flow cabinet for 30 minutes, then wrapped in foil, sealed in plastic and stored at 4°C for no longer than 2 days before use.

Figure 2.1 Plate format for chemotherapeutant testing

<i>Paramoeba</i> sp. ‘AGD’ Dilution					
Neat	1:4	1:16	1:64	1:256	
					5µg/ml
					10µg/ml
					15µg/ml
					20µg/ml
					30µg/ml

Chemotherapeutant Dilution

Growth inhibition assay

Repli-dishes were seeded with 0.1ml of *S. maltophilia* suspension (Appendix 2.1), added to each well. Up to five potential anti-amoebic chemicals were tested at one time, as well as negative and any solvent controls. All tests and controls were performed in duplicate. Actively growing *Paramoeba* sp. 'AGD' were harvested from plate cultures with 5-10ml of SSW, and the total number of cells estimated the number using a haemocytometer (Appendix 5). A suspension containing 5,000 *Paramoeba* sp. 'AGD' per ml was prepared, and diluted 1:4, 1:16, 1:64 and 1:256 from the neat preparation in SSW. Neat and diluted *Paramoeba* suspensions were added to the wells of the repli-dishes, 0.1ml per well, as shown in Figure 2.1. The plates were covered with aluminum foil, sealed in a plastic bag, and incubated for 2 weeks at 20°C.

Prior to scoring plates were dried in a Class II biological safety cabinet for between 30 and 60 minutes, and examined under a dissecting microscope for growth or survival of *Paramoeba* sp. 'AGD'. Growth was scored using the following scale:

- ++++ Almost complete coverage of the surface of the well with cells
- +++ 50% to 75% coverage of the well surface with cells
- ++ 25% to 50% coverage of the well surface with cells
- + less than 25% coverage of the well surface with cells.
- ± less than 10 cells on the well surface
- no cells observed on the well surface

The MIC was determined as the chemical dilution which showed little (\pm) or no growth, compared to the control plate (no chemical). The test was considered invalid if the control plate showed little or no growth. Chemicals that utilised solvents that later were found to be anti-amoebic themselves were solubilised with an alternative solvent and the growth inhibition assay repeated.

Wells with little or no growth of *Paramoeba* sp. ‘AGD’ due to the inhibitory effect of the chemical usually had a film of unconsumed *S. maltophilia*. Some compounds were found to be inhibitory to *S. maltophilia*, causing a reduction in the density of bacteria on the agar. In those cases where a bacterial film was not seen, the test was repeated using a different bacteria such as *E. coli* (Appendix 2.1) or *Flexibacter maritimus* (Appendix 2.2), resistant to the test compound.

2.9.3 Contact inhibition assay

This method was developed to determine the amoebicidal activity of a compound or treatment in a short-term aqueous exposure, based on the method used by Robinson *et al* (1990). This method was used to identify amoebicides that could potentially be used in a bath to treat fish with AGD. Table 2.1 shows the steps undertaken in the contact inhibition assay.

Exposure to chemical or treatment

Actively growing *Paramoeba* sp. ‘AGD’ were harvested from agar plates with SSW and the density adjusted to approximately 15,000 cells per ml. A total of 20 ml of cell suspension was required for each test chemical and a further 20ml for each control. Amoebicides were prepared as described in Method 2.9.1 and diluted to the desired concentration in SSW. The cell suspension was mixed vigorously with a vortex to ensure a homogenous suspension. For each test and control, 10ml of the cell suspension was dispensed into two separate sterile centrifuge tubes, and centrifuged at 1000g for 15-20 minutes. After centrifugation 9ml of supernatant was removed and the pellet resuspended in 9ml of the appropriate chemical/treatment or SSW (negative control). The cell suspension was mixed with a vortex mixer, and then aspirated through a 19-gauge needle, to break up any cell aggregates.

Immediately after resuspension, 2 ml from each of the duplicate negative controls was removed, and placed into sterile 10ml centrifuge tubes. The cells were washed by adding 8ml of SSW to each tube, mixing on a vortexer and centrifuging as described. After centrifugation 8 ml of supernatant was removed and the washing procedure repeated. After washing the pellet was resuspended in the remaining 2 ml of SSW, and further homogenised by aspirating the cells through a 19 gauge needle. This sample of cells represented the time=0 number of viable *Paramoeba* sp. 'AGD' in the sample.

Incubation of the cells in the chemical/treatment and in SSW (negative control) was continued at room temperature for 4 hours. After incubation 2ml samples were taken and washed as described; these samples represented the number of viable *Paramoeba* sp. 'AGD' after 4 hours exposure. In some experiments the minimum exposure time was determined by reducing the incubation period with the amoebicide. All contact inhibition assays were conducted at room temperature, which was always below 20°C.

The viable number of *Paramoeba* sp. 'AGD' in the samples was determined by the MPN method (below).

Viable count - Most Probable Number (MPN)

Samples were serially diluted (3 fold dilution series) in SSW and each dilution inoculated onto 10 replicate wells of a 25 well agar plate, Repli-dishes, seeded with *S. maltophilia* (Appendix 2.1). Plates were incubated for 14 days at 20°C. After incubation the number of wells, per dilution, were scored as positive for growth and negative for no growth. The viable number of *Paramoeba* sp. 'AGD' was calculated using an algorithm based on the process described by Parnow (1972), available on the statistical package, Genestat 5, version 3.1, Procedure: Dilution. All MPN counts were performed in duplicate. This assay is an adaptation of the most probable number (MPN) method used extensively to estimate the numbers amoebae in freshwater (Robinson *et al*, 1990).

Method: Repli-dishes were prepared by adding 1.5ml of MYA (Appendix 1.3) to each well, and the surface inoculated with 0.1ml of *S. maltophilia* (Appendix 2.1), the nutrient supplement. Each 2ml sample collected before and after exposure was serially diluted in a 3 fold dilution series, by transferring 750µl of the sample into 1.5ml of SSW and continuing until a dilution of 1:2187 was reached, comprising 7 dilutions and the undiluted neat sample. Ten wells of the Repli-dishes were inoculated with 0.1ml of each dilution, from each test and control, for each sampling period. Thus each test or control occupied 8 sets of 10 wells, representing the 8 dilutions and 10 replicates. After inoculation the plates were sealed in plastic bags to prevent the wells from drying out, and incubated for 2 weeks at 20°C. After incubation, the plates were dried in a Class II Biological Safety Cabinet and examined under a dissecting microscope for growth of *Paramoeba* sp. ‘AGD’. Wells were scored as positive or negative for growth, regardless of the density of growth. Growth was expressed per dilution, per test, as a score out of 10. A score of 10 relating to 10 wells with growth for a particular dilution, as per the example below:

Neat	1:3	1:9	1:27	1:81	1:243	1:729	1:2187
10	10	10	10	6	4	0	0

The number of viable *Paramoeba* in the samples was calculated by the MPN method using the Genestat 5 (Version 3.1, Procedure: Dilution) statistical package. The number of viable amoebae in the negative control sample after 4 hours exposure was compared with the number of viable cells exposed to the chemical/treatment. The mean for each control and test was calculated, and the reduction expressed as a percentage of the control. The reduction was considered significant only if the confidence limits did not overlap (for a more detailed explanation see Chapter 5). Chemicals/treatments that showed significant reduction were considered amoebicidal. Where a significant reduction was observed in the negative control samples at time=0 hours and at time=4 hours the assay was repeated, to exclude the possibility of a contaminant or high room temperature causing a reduction in the number of viable *Paramoeba* sp. ‘AGD’.

Table 2.1: Schematic showing the steps of the contact inhibition assay

PREPARATION	<ul style="list-style-type: none"> ■ Prepare chemicals (Method 2.9.1) ■ Prepare malt yeast agar repli dishes ■ Seed malt yeast agar plates with <i>S. maltophilia</i>
EXPOSURE TO CHEMICAL OR TREATMENT	<ul style="list-style-type: none"> ■ Harvest <i>Paramoeba</i> sp. 'AGD' and adjust to 15,000 cells/ml ■ Suspend cells in chemical/treatment or SSW (control) ■ Remove 2ml sample from control to act as time=0 hours exposure ■ Expose <i>Paramoeba</i> to chemical/treatment or SSW for 4 hours* ■ Remove 2ml samples from chemical/treatment and SSW control at time=4 hours exposure ■ Wash all samples in SSW to remove chemical/treatment
VIABLE COUNT MOST PROBABLE NUMBER	<ul style="list-style-type: none"> ■ Serially dilute all samples 1:3 to 1:2187 ■ Inoculate 10 wells of malt yeast agar repli dishes with 0.1ml of each dilution of each sample (time=0 and time=4) ■ Incubate repli-dishes for 14 days at 20°C ■ Score the number of wells positive for growth for each dilution ■ Calculate the viable number of <i>Paramoeba</i> sp. AGD using the MPN algorithm ■ Compare the number of <i>Paramoeba</i> exposed to the chemical/treatment at time=4 to the number in the control at time=4 to determine the percent reduction (See Chapter 5)

* In some experiments this time was reduced to determine the minimum exposure time required for an amoebicidal chemical or treatment

2.10 Gill mucus antibody extraction

2.10.1 Gill perfusion

This method, adapted from Lumsden *et al* (1993), was used to obtain from gill surfaces uncontaminated mucus prior to extraction of antibodies for immunological investigations.

Fish were anaesthetised to surgical level anaesthesia with approximately 100mg/l benzocaine (Ross and Ross, 1984). Fish were observed to be at the surgical anaesthesia level when they were gilling (opening and closing their opercula) very slowly, did not respond to exposure to the air, or to touch. Once surgical level anaesthesia was achieved the fish were removed from the water and as much body mucus removed by wiping the fish with dry paper towels, so as not to contaminate the gill mucus with body mucus. The fish were then placed in a V-shaped cradle, lined with paper towels. Working quickly, as much blood as possible was removed from the caudal vein using a 21 gauge needle and syringe, and collected into 10ml centrifuge tubes. After removing the blood the tail was removed, using a serrated knife. The heart was exposed by dissection and approximately 10-20ml of EDTA-saline (Appendix 13.3) injected into the bulbus arteriosus of the heart with a 23g needle. The process was continued until the gills were completely blanched. The gills were then excised and weighed, so as to relate antibody reactivity to weight of the gills. The gills were then either immediately processed for mucus antibody extraction by placing into the extraction buffer (Appendix 13.1), or frozen at -20°C and processed later. Serum was extracted from the blood and stored as previously described (Method 2.5.4).

2.10.2 Antibody extraction from mucus

This method, adapted from Lumsden *et al* (1993) describes the method used to extract antibodies from gill mucus.

Gills were placed in approximately 40ml of extraction buffer, in 50ml polypropylene centrifuge tubes, and shaken moderately overnight at 4°C on an orbital shaker. Gill material from large fish were processed by dividing the gills into two or three tubes of extraction buffer. After incubation, the gills were removed and the extract centrifuged at 30,000g for 30 minutes at 4°C. The supernatant was decanted and dialysed (Cellu Sep regenerated cellulose tubular membrane, nominal filter rating: 12,000, Cat # 1215-33) for 48 hours at 4°C with gentle agitation on an orbital shaker, against 2.5 litres of double distilled water containing PMSF and sodium azide (Appendix 13.2). The dialysis solution was changed at least 4 times during this period. The dialysates were then centrifuged at 1000g for 15 minutes to remove any remaining debris and precipitates. The supernatant was again collected and lyophilised overnight in a Dynavac D5 (Dynavac, Melbourne, Australia) freeze dryer. The lyophilised samples were resuspended in 1.0ml of PBS (Appendix 8.2) prior to ELISA testing and then stored frozen at -20°C.

2.11 ELISA to detect *Paramoeba* antibodies in fish

This method was developed to determine the presence of antibody to *Paramoeba* in fish serum and mucus (see Chapter 7).

Paramoeba sp. 'AGD' antigen was prepared and the protein concentration determined as previously described (Method 2.5.1). Microtitre plates (Nunc, Maxisorp, Cat No. NUN4-64394) were coated 100µl per well with antigen diluted to 10µg/ml in bicarbonate coating buffer (Appendix 11.1) and incubating overnight at 4°C. After coating, the wells were washed once with R.O. water. The remaining binding sites on the plates were blocked with blocking buffer (Appendix 11.2) by

adding 200µl per well, and incubating at room temperature for 1 hour. After blocking the plates were washed twice in R.O. water, and the excess removed by inverting the plates on absorbent paper. Serum or mucus samples were diluted 1:100 in dilution buffer DB (Appendix 11.4), and 100µl added to each well. One positive and 3 negative serum control samples (in duplicate) were added to each plate to validate the assay and to calculate the cut-off value². Test samples were assayed in duplicate. Plates were incubated at room temperature for 90 minutes, in a moist chamber. After incubation the plates were washed 3 times in ELISA wash buffer EWB (Appendix 11.3), as previously described. One hundred microlitres of mouse monoclonal anti-rainbow trout antibody (N. Gudkovs, Australian Animal Health Laboratory, CSIRO, Geelong), diluted 1:1500 in DB, was added to each well and incubated at room temperature for 1 hour. Plates were washed 3 times in EWB, as previously described. To each well, 100µl of conjugate, anti-mouse immunoglobulin conjugated to horseradish peroxidase (Silenus, Cat No. 985033020), diluted 1:2000 in DB, was added and incubated at room temperature for 1 hour. Plates were washed 3 times in EWB, as previously described and 100µl of TMB substrate (Appendix 11.6) was added to each well and incubated for approximately 10 minutes at room temperature, in the dark. The reaction was stopped by the addition of 100µl of stop buffer (Appendix 11.7) to each well. The optical density was measured at a wavelength of 450nm with a reference wavelength of 630nm.

The cut-off was determined by calculating the mean of the negative control optical densities plus 2 standard deviations. Samples with optical density readings greater than the cut-off were considered positive.

Control sera: Negative control sera were obtained from freshwater fish with no prior exposure to *Paramoeba* sp. 'AGD'. Positive control sera, produced by the inoculation of fish with *Paramoeba* sp. 'AGD' antigen (Method 2.5.3), were included as an internal control to validate the assay.

Adsorption: All serum and mucus samples were pre-adsorbed against *S. maltophilia* (Appendix 12), as some reactivity to *S. maltophilia* was observed in serum from sea-going Atlantic salmon.

² Mucus controls were unable to be included, as the method used to extract antibodies from mucus (Method 2.10.2) had not been validated for use on gill mucus from seawater fish. However, serum and mucus samples were tested together on the same plates to allow direct comparison of the results. For further explanation see page 283 (Chapter 7).

Chapter 3. Isolation and verification of the pathogen

3.1 INTRODUCTION

Roubal *et al* (1989) reported a *Paramoeba* species as the pathogen associated with amoebic gill disease (AGD) in sea-farmed Atlantic salmon in Tasmania. Gill disease associated with *Paramoeba pemaquidensis* had also been reported in sea-farmed salmonids the USA (Kent *et al*, 1988) and Ireland (Rodger and McArdle, 1996; Palmer *et al*, 1997); and in sea-cultured turbot in Spain (Dyková *et al*, 1998c). Most of the evidence suggesting a pathogenic role for this species in AGD came from histopathological studies, where amoebae identified as *Paramoeba* could be observed associated with gill damage.

The pathogenicity of *Paramoeba* has not been convincingly demonstrated. Previous attempts to challenge naïve fish with this organism failed to produce disease (Kent *et al*, 1988; L. Searle, Pers. Comm.). Marine amoebae belonging to genera other than *Paramoeba* have been isolated from fish with AGD (P. Statham and P. Larson Pers. Comm.; Dyková *et al*, 1999) but the role of these other marine amoebae in disease is not clear. At the time of this study, no isolates of *Paramoeba* species from fish with AGD had been successfully maintained in Australia.

The objectives of this portion of work were to isolate and characterise amoebae from fish with AGD and to determine the pathogenic role of the cultured amoebae, by their association with gill damage in fish with AGD. Once the relationship of the isolates to the disease was understood it would be possible with certainty to: undertake pathogenicity trials; determine the inhibitory effects of proposed treatments; and develop rapid sensitive assays to detect amoebae in clinical material.

3.2 METHODS

3.2.1 Isolation and characterisation

Isolation sites: The main risk period for AGD in Atlantic salmon occurs when they are transferred into the sea in October, the fish are at continued risk over the summer and early autumn period usually until April. Isolation of amoebae from Atlantic salmon gills was therefore undertaken late in October, approximately two weeks after acclimatisation. To increase the chances of isolating the amoebae, particularly the causative agent of AGD, fish were sampled from three different farms. In previous years, most isolation work had been undertaken at Dover (farm A) but whilst AGD did occur frequently at this site, the pattern was not always typical due to the affects of freshwater flushes from the nearby river. Two fully marine sites designated farm B and farm C, were also sampled in this study, both sites not usually being subject to freshwater flushes.

Sampling Regime: Based on previous years experience, three ‘waves’ of infection were expected before the end of the risk period in April. The first ‘wave’ of infection was predicted to run from October to late December, when most farms began prophylactic bathing. Samples were collected in the early, middle and late stages of the infection periods (Table 3.1). Samples taken late in infection were collected during freshwater bathing, wherever possible both pre-bathed and post-bathed fish were selected. Up to 10 fish from each site were sampled per collection.

The second ‘wave’ of infection was of a much shorter duration lasting approximately six weeks. Samples were taken during the middle and late periods of infection from farm A, whilst only late samples were taken from farms B and C. Samples taken late in infection included mostly pre-bathed fish, although a small number of post-bathed fish were also sampled.

Samples were collected from farm A during ‘wave’ 3, from a pen of control fish that had never been bathed in freshwater. No other farms were sampled during ‘wave’ 3.

Table 3.1: Summary of farm sampling times

'Waves' of Infection	Date	Farm	AGD Time Period
1	28 October 1991	C	Early
	29 October 1991	B	Early
	12 November 1991	A	Early
	27 November 1991	C	Middle
	21 December 1991	A	Middle
	3 December 1991	B	Middle
	16 December 1991	B	Late and post bathing
	17 December 1991	C	Late
	19 December 1991	A	Middle
2	16 January 1992	A	Middle
	29 January 1992	B	Late and post bathing
	4 February 1992	A	Late
	14 February 1992	C	Late
3	3 March 1992	A	Late*

* Fish never bathed.

Gross gill health: During sampling, gills were examined for any gross signs of damage; including increased levels of mucus, mucous patches, the presence of small parasitic copepods (isopods) (Figures 3.1 and 3.2), and the overall severity of AGD assessed by scoring the number of mucous patches (see Method 2.6.1).

Isolation techniques: Unless otherwise stated, all gill samples were cultured on sea-water agar plates (Appendix 1.5) seeded with *Flexibacter maritimus* (Appendix 2.2). To inhibit overgrowth by bacteria a “cocktail” of antibiotics (Appendix 3.1 and 3.2) were added to the molten agar at a ratio of 1:1000. If mucous patches were observed on the gill, these areas were selected for culture. Antibiotics (Appendix 3.1 and 3.2) were also added to sterile sea-water (Appendix 1.1) and sterile reverse osmosis (R.O.) water (Appendix 1.2) used in the isolation procedures, at a ratio of 1:1000. The plates were observed over an eight week period for migration of amoebae from the gill sample, using a dissecting microscope with adjustable sub-stage lighting and glass stage, magnifying to at least X50.

Cultures were obtained using five methods of isolation to reduce selectivity and maximise the likelihood of isolating any amoebae associated with the gills.

- (i) Pieces of gill, approximately 2.5cm², were rinsed in sterile sea-water containing antibiotics and placed onto seeded sea-water agar plates.
- (ii) Pieces of gill were placed directly into 2cm diameter wells, cut into the seeded sea-water agar plates. Sterile sea-water containing antibiotics was placed in the wells to cover the samples.
- (iii) Single gill arches were washed with 10 ml of sterile sea-water containing antibiotics, for 1-3 hours on a shaker table. Gill tissue was removed, the extract concentrated by centrifugation (1000g) for 15 minutes, and 500µl of the concentrate inoculated onto the centre of seeded sea-water agar plates. Where no shaker table or centrifuge was available on site, the samples were stored in the sea-water with antibiotics and processed in the laboratory within 5 hours of collection.
- (iv) Single gill arches were washed in sterile R.O. water (Appendix 1.2) containing antibiotics, to mechanically remove amoebae; a process believed to occur during freshwater bathing of fish. Gill material was processed as in isolation method 3 (above).
- (v) Freshwater or euryhaline amoebae possibly involved in AGD were isolated on seeded freshwater agar plates (Appendix 1.4). Pieces of gill were placed directly into 2cm² diameter wells cut into seeded freshwater agar plates. Sterile R.O. water containing antibiotics was placed in the wells to cover the samples. The plates were observed for migration of amoebae out of the wells and/or the presence of cysts, a characteristic stage in the life cycle of freshwater amoebae. This method was discontinued after the first period of infection.

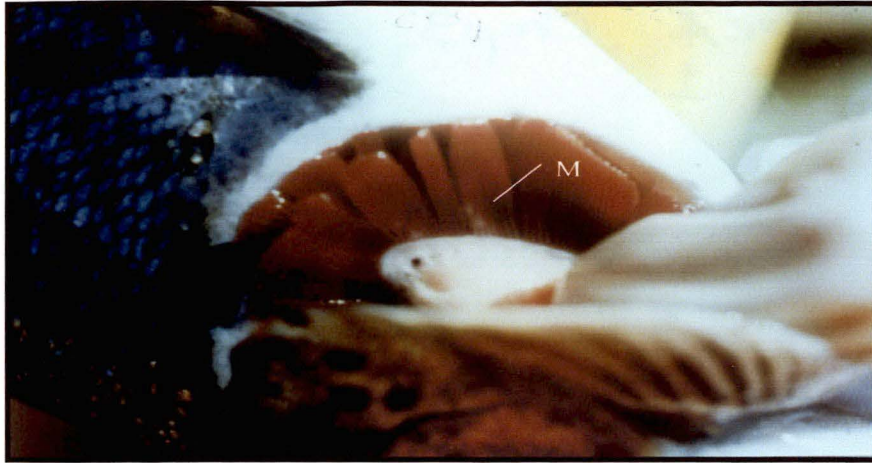


Figure 3.1: Atlantic salmon gill showing the occurrence of small mucous patches (M), indicative of early AGD.

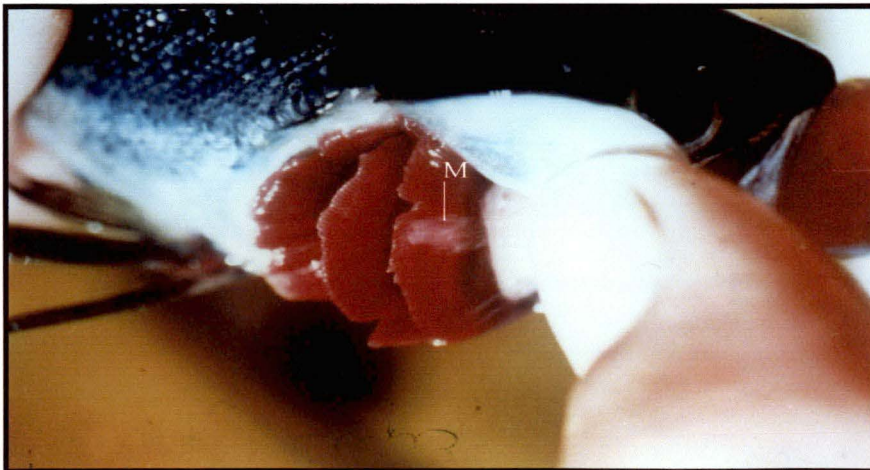


Figure 3.2: Atlantic salmon gill showing an extensive mucous patch (M), indicative of moderate to severe AGD.



Figure 3.3: Atlantic salmon gill showing the presence of a large isopod

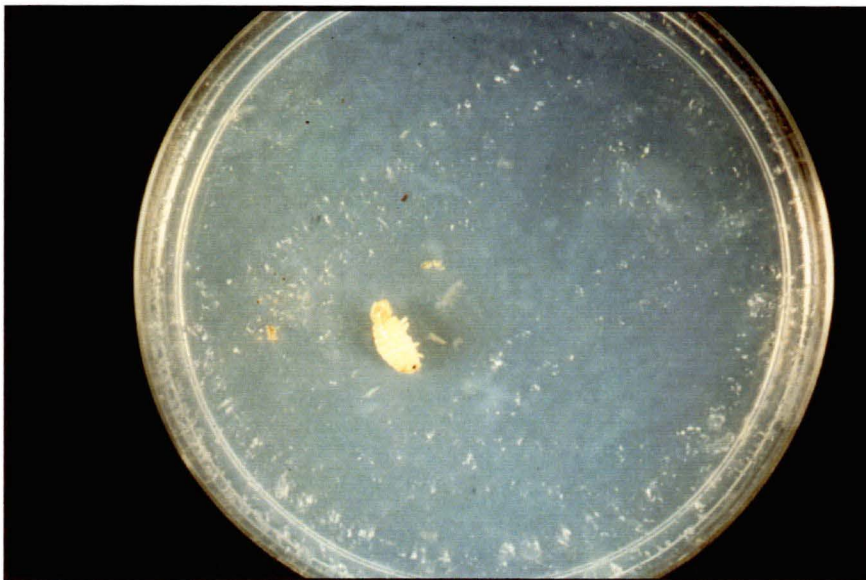


Figure 3.4: Isopod and isopod trails on an agar plate

During the sampling programme, isopods were observed at some sites attached to gills. The incidence of isopods appeared to increase during the ‘waves’ of infection. To determine if isopods could act as a reservoir of amoebae, isopods were placed onto seeded sea-water plates and allowed to crawl across them. The plates were observed for the migration of amoebae from the isopod trails (Figure 3.4).

Subculturing and purification: Amoebae were cloned (Method 2.2.2) onto either sea-water agar or freshwater agar plates, and subcultured as described in Method 2.1.2. Purified isolates were initially maintained on agar plates seeded with *E. coli* (Appendix 2.1), prior to identification and inoculation into rabbits for antisera production. *E. coli* was used for maintenance rather than *F. maritimus* to ensure there were no non-specific reactions when gill sections were probed with anti-amoeba antisera. *F. maritimus* is known to occur both as a pathogen and as normal flora of gills from sea run Atlantic salmon. Later these purified isolates were maintained on malt yeast agar (Appendix 1.3) seeded with *Stenotrophomonas maltophilia* (Appendix 2.1), a bacterial substrate later found to be superior to *E. coli*, especially as a food source for *Paramoeba* species. Contamination of cultures was avoided or treated as described in Method 2.1.4. Old or dying cultures were revived as described in Method 2.1.5.

Identification: Identification of isolates was undertaken in collaboration with Dr Bret Robinson, State Water Laboratory South Australia. Purified amoeba isolates were identified to the generic level, based on morphological characteristics (Page, 1983; Robinson *et al*, 1990). In some cases, identification of mixed cultures was also attempted. Page (1983) reported that “generic diagnoses carry a higher degree of certainty than do species diagnoses” in marine gymnamoebae, and that non-morphological approaches would be necessary to distinguish individual species, thus identification of most isolates to the species level was not attempted, unless an outstanding characteristic was identified.

Preliminary observations were carried out using a dissecting microscope, with adjustable sub-stage lighting and a ground glass diffuser at a magnification of X50. Plate cultures were examined to identify areas of growth. A coverslip was placed on top of each area to be examined. More detailed observations were made using a compound microscope with a long focal length condenser, to allow plate cultures to be placed directly onto the stage, and examined under low X100 and high X400 or X1000 (oil immersion) magnifications (both phase contrast) for both trophozoites and cysts (if present).

The following characters were used in the generic identification of amoeba isolates, based on the characteristics described by Page (1983).

Locomotive form (trophozoite): Isolates were observed to be either limax ("slug like") that is, cylindrical with a rounded anterior end, longer than broad; or compressed or flattened, broad amoebae with conspicuous hyaloplasm. Limax amoebae were further divided into those with eruptive locomotion and those where locomotion is more steadily flowing. Compressed or flattened amoebae were distinguished using the following criteria: outline; oblong, oval, flabellate or spatulate; sub pseudopodia, present or absent, long or short, branched or unbranched; or the presence of uroidal filaments (filaments formed at the posterior end of the amoeba resulting from adherence to the substrate as the amoeba moves).

Size: Measurements of length and breadth in microns (μm) were taken of the locomotive form of the amoebae.

Cytoplasmic inclusions: Isolates were examined for the presence of a parasome, a complex DNA containing body found in *Paramoeba* species in addition to the nucleus, and also for any other distinguishing inclusions.

Nuclear Structure: The structure and location of the nucleolar material was determined. The nucleolus could be seen as a single central body or as two or more lobes in a parietal (peripheral) position.

Cysts: The morphology of any cysts was observed. Cysts are useful for the identification of freshwater and soil amoebae but true marine amoebae are not known to form cysts.

Floating form: The morphology of amoebae suspended in sea-water was observed: the presence or absence of pseudopodia, and the appearance of the pseudopodia, slender, tapering or blunt.

Flagellate form: Demonstration of a flagellate stage was used for the identification of cyst-forming amoebae. Trophozoites were suspended in 100µl of 2M Tris buffer (pH 7.2) and examined over a period of 2-24 hours for transformation into flagellates (Robinson *et al*, 1990).

3.2.2 Verification of the pathogen

Gill collection: Gill samples were collected in conjunction with the isolation work for microscopic assessment of gill health, labelled antibody staining, and to determine at what stage gill damage occurs and amoebae are isolated. Gill samples were collected as described in Method 2.6.2. Three fixation methods were used to collect and store gill samples, based on their respective perceived benefits.

- (i) Pieces of gill were collected into 4-10 ml of isotonic formalin (Appendix 6.2). Sections were later trimmed and mounted in wax. Formalin is a slow acting fixative used to ensure suitable fixation of gill tissue, however amoebae are more likely to be lost from these sections.
- (ii) Pieces of gill were collected into 4-10 ml of isotonic Davidson's fixative (Appendix 6.4). Sections were trimmed and mounted in wax approximately 24-48 hours later. Davidson's fixative is a rapid fixative used to improve the fixation of amoebae in gill tissue, allowing morphological examination of amoebae *in situ*.

- (ii) To ensure the integrity of amoebic antigens, necessary for immunostaining, up to 2 gill rakers were sealed in plastic and snap frozen in liquid nitrogen. Sections were stored at -70°C prior to sectioning using a cryostat. It was not known whether formalin or Davidson's fixatives could cause adverse changes to the amoebic antigens in the preserved tissue.

Microscopic gill health: Gill health was assessed using sections cut from gill tissue fixed in formalin and Davidson's fixatives, and stained with haematoxylin and eosin (H&E) stain. The sections were observed microscopically for the presence of amoebae, mucous cells, nodules of lymphoid concentration, fusion of lamellae, hyperplasia and other abnormalities or signs of AGD (as described in Method 2.6.2). Isopods fixed in formalin and Davidson's fixatives were also cut, stained and examined microscopically. Isopod sections were examined for the presence of amoebae, parasites or commensals that may contribute to AGD. Microscopic examination of the sections was conducted by Dr Judith Handler at the Fish Health Unit, Department of Primary Industries, Water and Environment, Launceston, Tasmania.

Antiserum production: Antiserum was obtained by intravenous injection of rabbits (as described in Method 2.5.2) with some slight variations. No adjuvant was used. All amoebae were maintained on sea-water agar plates seeded with *E. coli* prior to inoculation into rabbits. Serum was prepared as described in Method 2.5.4. The amoebae chosen for antiserum production were selected based on the frequency with which they were isolated, and on previous observations of the morphology of the amoebae associated with AGD (Kent *et al*, 1988; Roubal *et al*, 1989; Munday *et al*, 1990).

Antisera to *Paramoeba* (PA-016) and *Flabellula* (FLB-004) were prepared using sonicated antigen (Method 2.5.1). Injections ranging from 300,000 to 3 million amoebae were administered at 3 day intervals for approximately 3 weeks. Antisera was collected after 4 weeks.

Antiserum to *Platyamoeba plurinucleolus* (UQ-1) was prepared using sonicated antigen, with injections ranging from 500,000 to 1.4 million amoebae administered at 3 day intervals for 5 weeks. Antiserum was collected after 6 weeks. Antiserum to a *Vannella/Platyamoeba* mix (MP-1) was prepared in 1989 using whole antigen (prepared by Pat Statham, DPIWE, Launceston, Tasmania). Both the *P. plurinucleolus* and *Vannella/Platyamoeba* mix had been collected from fish with AGD and were thought at the time to be the pathogens involved.

To determine the specificity of the antibody, pure cultures of four corresponding amoeba isolates were reacted with each of the four antisera using a fluorescein labelled anti-rabbit IgG. Amoebae were collected from plate cultures, removed from the surface with sterile sea-water, smeared onto clean microscope slides, air dried and then fixed by passing over a Bunsen flame. The IFAT was performed on these slides as described in Method 2.6.3. Antisera were tested at a dilution of 1:100. A negative control of phosphate buffered saline was included¹. Each isolate was tested in duplicate. Slides were examined by epifluorescent UV microscopy for the presence of brightly fluorescing yellow/green amoebae, and scored as either positive or negative (no fluorescence).

To determine if the antiserum to PA-016 was specific to all the *Paramoeba* isolates collected, surviving *Paramoeba* isolates were reacted with PA-016 antiserum using the method above. To further ensure the specificity of the *Paramoeba* antiserum it was tested against a total of 42 amoebae, not identified as *Paramoeba* sp., isolated during this study.

¹ Normal rabbit serum was also tested as a negative control, resulting in no background fluorescence. Although an appropriate negative control it offered no appreciable benefits over phosphate buffered saline, which was more readily available.

Verification by immunostaining

Immunostaining is a sensitive immunological technique that can be used to detect specific organisms in tissue (Polak and Van Noorden, 1984). A two-stage methodology was employed to detect the amoebae in gills:

- (i) The rapid immunofluorescence (IF) method was used to screen samples for the presence of *Paramoeba*, *Flabellula*, *Platyamoeba plurinucleolus* and *Platyamoeba/Vannella* sp. in fixed gill tissue samples. This procedure only detects the presence of the target organism; the gill tissue was not visible. A small number of frozen gill samples were also tested. Sections of formalin fixed brain tissue, from rainbow trout infected with a *Streptococcus* species, were stained with an anti-streptococcus antibody, to validate the method.
- (ii) The immunoperoxidase (IP) method stains the target organism for examination by light microscopy, allowing it to be viewed in relation to the tissue damage. Sections previously found positive by immunofluorescence were immunostained using this method.

Preparation of fixed gill samples: Sections of formalin or Davidson's fixed gill sections were cut at approximately 5-10µm, mounted on slides coated with Poly-L-Lysine® (Appendix 7.1), and de-paraffinised to remove the wax (Appendix 7.2). Some sections were digested with 0.1% pronase in PBS, a protease, to expose antigenic sites that may have been inhibited by chemical fixation.

Preparation of frozen gill samples: Frozen gill sections were defrosted, trimmed and mounted in O.C.T. solution (Tissuetek Inc., U.S). The mounted sections were then refrozen and cut using a cryostat. Sections were mounted on slides coated with Poly-L-Lysine® and kept refrigerated prior to testing.

Immunofluorescence: A selection of gill samples collected in formalin and Davidson's fixative, known to be positive for amoebae by histological examination, were immunostained using antisera made to the four amoeba isolates and a fluorescein labelled anti-rabbit IgG (Method 2.7.1). The gill tissues screened by this method came from a variety of sources. Table 3.2 shows the samples screened by the four antisera.

Between 29 and 37 gill samples were screened with all four antisera: some of these gill samples were collected during this study; the remaining samples came from a library of gill samples submitted to the Fish Health Unit (DPIWE, Launceston, Tasmania) from 1985 to 1991, for histological diagnosis of AGD. All samples were known to be positive for amoebae by histological examination. Sections taken from two isopods were also screened with the four antisera. All sections were examined for fluorescent amoebae, and wherever possible compared to the same section stained with haematoxylin and eosin (H&E) by light microscopy.

Twenty-two additional sections, with few or no amoebae by H&E staining but with gill damage consistent with AGD, were tested using antisera to the *Paramoeba* sp., in an attempt to determine the sensitivity of the immunofluorescence method.

Also included in the gill selection were five samples collected from harvest sized fish during the winter of 1992, from a population with clinical signs of AGD. AGD had never been observed in harvest sized fish prior to this year, and is not thought to occur due to the hypothesised development of protective immunity (B. Munday, Pers. Comm.). A number of gill samples collected during suspected AGD outbreaks in New Zealand in 1989 and France in 1993 were also immunostained using antiserum to *Paramoeba*.

A small number of frozen gill samples collected during this study, with histological evidence of AGD, were screened with antiserum to *Paramoeba* sp. A total of 15 frozen gill samples were screened, from two sites and sample dates. One isopod was also sectioned and screened

Table 3.2: Table representing the origin and number of samples tested by immunofluorescence, with each antisera

	Antisera			
Histology samples	<i>Paramoeba sp.</i>	<i>Flabellula sp.</i>	<i>Platyamoeba/Vannella sp.</i>	<i>Platyamoeba plurinucleolus</i>
Library of AGD gill sections*	37	29	32	31
Isopod sections	2	2	2	2
AGD sections with few or no amoebae	22	ND	ND	ND
Gill sections from harvest-sized fish	5	ND	ND	ND
Gill sections from New Zealand	3	ND	ND	ND
Gill sections from France	2	ND	ND	ND
Frozen gill and (isopod) sections	15 (1)	ND	ND	ND

* gill sections collected during this study and routine submissions from 1985-1991, positive for amoebae by histology

ND = not done

Immunoperoxidase: A selection of gill samples found positive for amoebae by immunofluorescence, were immunostained with antiserum to *Paramoeba*, using the immunoperoxidase method (Method 2.7.2). The sections were counterstained with haematoxylin (Appendix 8.8), and the slides examined for dark red/brown stained amoebae against a pink background. To intensify the peroxidase reaction, some sections were treated with 1% osmium tetroxide in PBS prior to counterstaining with haematoxylin.

Methyl green was tested as an alternative counterstain to haematoxylin in an attempt to increase the contrast between the amoebae and the gill section. In a further attempt to increase the contrast some sections were also immunostained using an alkaline phosphatase labelled reagent (Method 2.7.3), slides were examined for blue amoebae against a pink background.

3.2.3 Additional Investigations

Long term preservation: To maintain the genetic integrity of the amoeba isolates collected during this study, and to protect against the selection of laboratory adapted strains, a number of methods were investigated for long term storage of isolates. Previous experience with a *Platyamoeba/Vannella* mix of isolates suggested that freezing at -70°C might be successful in preserving marine amoebae (P. Statham, Pers. Comm.).

A preliminary investigation was undertaken using a *Platyamoeba plurinucleolus* isolate (UQ 1). Aliquots of 1ml, each containing 6000 amoebae were suspended in sterile sea-water (SSW) and SSW with 2%, 5% and 8% DMSO. All samples were immediately frozen at -70°C. The aliquots of each of the four suspensions were checked at 2 weekly intervals for 6 months for viability. Samples were thawed rapidly in a water bath at 37°C. Amoebae were observed by phase contrast microscopy for cellular integrity and then plated out onto freshly seeded malt yeast agar plates, incubated at 20°C, and growth examined at 3 and 5 days.

Additional techniques were investigated to freeze *Paramoeba* isolates. Two *Paramoeba* isolates (PA-016 and PA-002) were frozen using the method investigated for *P. plurinucleolus* described above, with SSW alone, and SSW with 5% DMSO. These isolates were also suspended in SSW and snap frozen in liquid nitrogen before being placed at -70°C. Recovery was attempted 4 days after freezing as described above and also by defrosting at 20°C.

To increase the success rate of freezing and retrieval of amoeba isolates and the range of genera able to be frozen, a method used to successfully freeze freshwater amoebae, *Acanthamoeba* and *Naegleria* species, was modified for marine species (Robinson *et al*, 1990). This method (Method 2.4.1) involved concentrating the amoebae in a cryopreservation medium (Appendices 9.1 and 9.2) containing DMSO and made up with SSW for fully marine amoebae, freshwater for freshwater isolates and 50% SSW for euryhaline amoebae. Cyst-forming amoebae, were allowed to encyst before processing. Cultures were retrieved from -70°C by rapid thawing at 37°C, resuspended into the appropriate salinity water and then inoculated onto the appropriate seeded agar plate, as described in Method 2.4.2. Attempts were made to cryopreserve all surviving cultures collected during this study. Cultures were examined for viability after 2-3 weeks storage at -70°C, and selected cultures periodically examined for viability over a 3½ year period.

Refining isolation techniques: A technique using an ammonium chloride wash, to remove amoebae from the gills of fish, was developed by Jones (1988) to effectively remove *Paramoeba* from the gill tissue of AGD affected fish. Initial work undertaken in the present study had found that this technique was amoebicidal to some amoebae. This technique was therefore re-examined and modified in this phase of the study in an attempt to increase the number of *Paramoeba* species that could be isolated from fish gills.

To determine the effectiveness of this technique the gills from three AGD infected fish were processed as described in (Method 2.2.1). Gills were washed in a solution of ammonium chloride in sea-water containing antibiotics (Appendix 3.3). After washing the gill tissue was removed and the wash concentrated to 1-5ml by centrifugation (1000g for 15-20 minutes). The concentrate was then resuspended by vigorous mixing and smeared onto Poly-L-lysine® coated slides (Appendix 7.1). Slides were then air-dried, and the presence of *Paramoeba* species determined by immunofluorescent antibody testing (IFAT) with *Paramoeba* antiserum (Method 2.6.3.). This concentrate was inoculated onto malt yeast agar plates (Appendix 1.3) seeded with *S. maltophilia* (Appendix 2.1), to determine the efficacy of this method in removing viable *Paramoeba* species and its efficiency for the isolation of *Paramoeba* species over other species of marine amoebae.

Confirmation of parasome: The presence of a parasome is a diagnostic characteristic of the genus *Paramoeba*. This feature can be easily observed by observing growing plate cultures using a compound microscope with a long focal length. The laboratory used in this study did not possess such a microscope, although one was available during the identification component of the study, hence other methods of confirming the presence of a parasome in suspected *Paramoeba* isolates were required. As compound microscopes with long focal lengths are a dedicated piece of machinery, not commonly available in most laboratories, a number of techniques were investigated in this study, to provide the means of definitively identifying *Paramoeba* species in both the field and the laboratory. This was considered an important aspect of all future work, to avoid the past confusion of *Paramoeba* species with other species of amoebae.

Two staining techniques were investigated, both being based on staining DNA, allowing the identification of more than one DNA rich body, a diagnostic feature of *Paramoeba* isolates. A staining technique, developed by Rogerson (1988) to rapidly detect nuclei and parasomes in marine gymnamoebae, was investigated for its ability to identify parasomes in our laboratory (as described in 2.3.2.). This method required the use of a microscope with epifluorescence filters and objectives. The second staining technique investigated involved a standard histological stain, Gomori's trichrome stain (Method 2.3.3), and required the use of a standard light microscope.

An alternative less complicated method not involving staining was also investigated, to allow definitive identification in laboratories with limited resources, such as field based laboratories. Amoebae were placed onto slides coated on one side with 2% agar (Appendix 4.1), the slides were then coverslipped and observed under a light microscope with phase contrast optics to X40 (see Method 2.3.1).

Preliminary identification of *Paramoeba* isolates: The growth characteristics of the cloned amoeba isolates collected during this study were carefully examined over a one year period to allow preliminary identification of *Paramoeba* and other selected genera. To observe the growth characteristics of cloned cultures, actively growing plates were observed under a dissecting microscope, with adjustable sub-stage lighting and a ground glass diffuser at X35 and X70 magnifications. Areas of active growth, referred to here as the growth front, and older growth, the area closest to the point of inoculation, were examined. The effect of the amount of bacterial substrate present on the plates, relating to the growth characteristics, was also examined. In this study *Paramoeba eilhardi* were not successfully maintained in culture and therefore the growth characteristic of this species could not be compared.

3.3 RESULTS

3.3.1 Isolation and characterisation

Gross gill health¹: Table 3.3 shows the results of the gross gill health of the fish sampled during this study. No signs of AGD were seen in samples taken early in ‘wave’ 1 of infection. Mucus levels and mucous patches increased during ‘wave’ 1 and this pattern was observed to increase during subsequent ‘waves’ of infection. Post-bathed fish showed lower mucus levels compared to pre-bathed ones in ‘wave’ 1; bathing also appeared to eradicate the mucous patches, but this was not observed in ‘wave’ 2 where the only effect of bathing was to reduce the number of patches. The number of isopods was observed to increase during the ‘waves’ of infection, particularly on the gills of fish sampled from farm A that had never been bathed in freshwater. A significant number of the fish sampled from farm A showed gills with large areas of primary lamellae missing, sometimes over a whole gill raker, and also the occasional appearance of gills with a “mushy” almost necrotic appearance (Figure 3.5). Overall, the level of AGD observed during the sampling period was mild to moderate; only in fish that had never been bathed was there evidence of a more severe infection. No significant mortalities due to AGD were reported from any of the farms sampled.

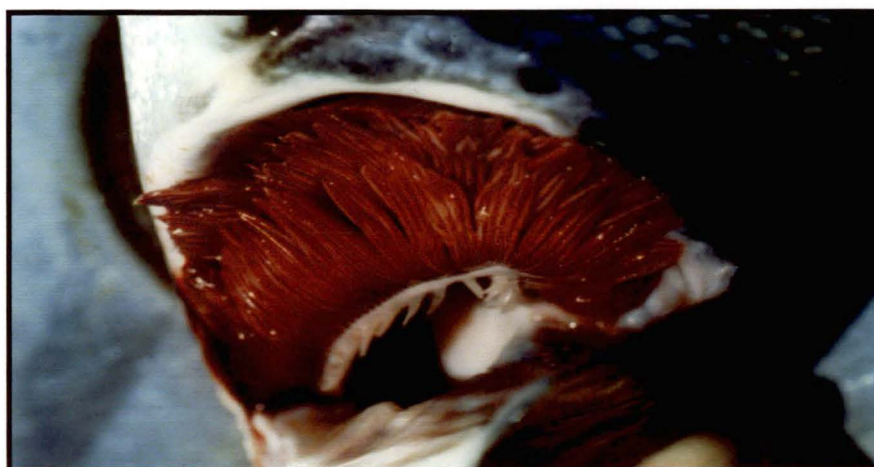


Figure 3.5: Atlantic salmon gill showing ‘mushy’ appearance and diffuse mucus.

¹Gross gill health refers only to obvious changes to the gill from normal, able to be seen by eye. The results of gill histopathology examination are reported in section 3.3.2.

Table 3.3: Gross assessment of gill health during sampling programme

Date	Farm	Number Sampled	Gross Signs of Disease			
			Mucous Patches	Mucus	Level of AGD	Isopods
‘Wave’ 1						
28 October 1991.	C	11	-	-	-	-
29 October 1991	B	10	-	-	-	-
12 November 1991	A	12	-	-	-	-
27 November 1991	C	10	-	+	-	-
2 December 1991	A	9	-	+	-	-
3 December 1991	B	10	+	++	+	-
16 December 1991	B	10	++	+++	++	+
		5 post-bathed	-	+	NA	+
17 December 1991	C	5	-	++	+	+
19 December 1991	A	10	-	++	+	+
‘Wave’ 2						
16 January 1992	A	10	++	+++	++	NR
29 January 1992	B	5	++	+++	++	+
		4 post-bathed	+	++	NA	+
4 February 1992	A	10	+	+++	++	+++
14 February 1992	C	12	++	+++	++	-
‘Wave’ 3						
3 March 1992	A	12	++	+++	+++	+++

- None/ Normal
+ Few (1-4 patches)/Slight
++ Moderate (5-9 patches)
+++ Many (≥ 10 patches)/Excessive
NA Not assessed as damage same as pre-bathed fish
NR Not recorded

Isolation: Approximately 680 cultures were prepared during the three ‘waves’ of infection. From these cultures, 61 amoeba isolates were successfully cloned and subcultured. A small number of amoeba isolates could not be successfully subcultured. Table 3.4 shows the efficiency of the isolation methods employed. The most successful isolation method was the sea-water wash, followed by the gill tissue method and isopod trails. The sea-water well and freshwater wash methods were relatively unsuccessful in isolating amoebae. One freshwater amoeba isolate was obtained using the freshwater well method, which was discontinued after the first wave of infection.

Most of the 61 isolates were grown from gill material collected late in ‘wave’ 1 and in ‘wave’ 2 and 3 (see Table 3.8). Few isolates were grown from early and middle sampling in ‘wave’ 1.

Table 3.4: Comparative efficiency of amoeba isolation methods

Isolation Method	Total number of cultures prepared	Number of isolates	Overall efficiency (%)
Gill Tissue	145	14	9.66
Well (sea-water)	145	6	4.14
Sea-water wash	145	21	14.48
Fresh water wash	145	2	1.34
Well (freshwater) ^A	92	1	1.09
Isopod trails	9-12 ^B	14 ^C	155 – 116.7
Total	681 - 684	61	8.92 - 8.96%

A This method was discontinued after the first ‘wave’ of infection
 B Exact number of cultures not recorded
 C Figure reflects more than one isolate cultured from primary culture plate

Identification: Of the 61 amoeba isolates, 33 were identified to the generic level. Two of the 33 were further identified to the species level because of their unique characteristic features. Identification of the remaining isolates was not been attempted due in large part to time constraints, however these isolates did not contain *Paramoeba* or *Flabellula* isolates as these were easily identified using the criteria used.

Seven genera of amoeba were identified (see Table 3.5). The vast majority of the isolates, 17 out of 33, belonged to either the genus *Platyamoeba* or *Vannella*. Separation of these two genera is difficult and relies heavily on morphological features, which were often hard to distinguish. Page (1983) commented “distinguishing from *Vannella* and *Platyamoeba* with the light microscope alone may be relatively easy or impossible, depending on the isolate”. For this reason the further identification of the *Vannella* and *Platyamoeba* isolates cultured in this study was not attempted; all future reference to these isolates will refer to them as *Platyamoeba/Vannella* isolates. This group contained a range of distinct morphological types, the size of the isolates ranged from very small ($\approx 5\mu\text{m}$) to large ($< 40\mu\text{m}$). Only two isolates from this group could be identified to the species level. These were *Platyamoeba plurinucleolus* identified by its characteristic nucleus with parietal chromatin.

Only six out of the 33 isolates were identified as *Paramoeba* sp. One of these was identified as *Paramoeba eilhardi*, the others are probably *Paramoeba pemaquidensis* (Figures 3.6 – 3.8) as previously described by Page (1980) and Kent *et al.*, (1988) (see Table 3.6). For the purposes of this study these isolates were referred to as *Paramoeba* sp. ‘AGD’. The remaining amoeba isolates were identified as belonging to the genera *Flabellula* (Figure 3.9), *Heteroamoeba* (Figure 3.10 and 3.11), *Acanthamoeba* and *Vexillifera*. Tables 3.7a and 3.7b were compiled using the features observed during the identification of these isolates, to aid the identification of amoebae from the gills of fish with and at risk of AGD.

Amoeba isolates belonging to the genus *Flabellula* were isolated from farm C only, during one sampling time. *Acanthamoeba* sp. and *Heteroamoeba* sp. were only isolated from farm A. None of the other commonly isolated genera were site specific (see Table 3.8).

Paramoeba were isolated from two farms, late in 'wave' 2 and 'wave' 3. Of the six *Paramoeba* isolates, four were cultured from isopod trails while the remaining two were cultured using the sea-water wash method (see Table 3.6). The *Acanthamoeba* species was isolated using the freshwater well method. There was no correlation between method of culture and isolation of the remaining genera.

Differences in growth speed and adaptation to the growth substrate were noted between the amoebae genera. Isolates identified belonging to the *Platyamoeba/Vannella* and *Flabellula* species were typically fast growing, adapting well to a change in growth medium from mixed flora (from primary isolations) and to *Flexibacter maritimus* and *E. coli* substrates. *Paramoeba* isolates were slow growing and initially difficult to adapt to axenic bacterial culture, *Paramoeba* cultures retained by Dr Bret Robinson at the State Water Laboratory, SA were eventually lost due to these difficulties (B. Robinson, Pers. Comm.). Diatoms were observed growing in conjunction with four out of the six *Paramoeba* isolates, but culture of diatoms as a growth substrate was not attempted. *P. eilhardi* could not be maintained in culture despite many attempts. The *Acanthamoeba* sp. isolate was cultured exclusively on freshwater agar containing *E. coli* as a growth substrate. The *Heteroamoeba* sp. was maintained on sea-water agar with *E. coli*. All other isolates were maintained for a period on sea-water agar seeded with *E. coli* as a growth substrate. After antiserum production all isolates except *Acanthamoeba* were maintained on malt yeast agar (Appendix 1.3) seeded with *S. maltophilia* as the growth substrate

Table 3.5: Genera of amoebae isolated from fish with or at risk of amoebic gill disease

Genera	Number of Isolates	Normal Habitat
<i>Acanthamoeba</i>	1	Fresh/Euryhaline
<i>Flabellula</i>	7	Sea
<i>Heteroamoeba</i>	1	Brackish
<i>Platyamoeba/Vannella</i> ¹	13	Sea
<i>Platyamoeba plurinucleolus</i> ²	2	Sea
Small <i>Platyamoeba</i> ³	2	Sea
<i>Paramoeba</i>	6	Sea
<i>Vexillifera</i>	1	Sea

1 Genera difficult to distinguish by morphology alone

2 Isolate speciated due to its characteristic nucleus

3 These distinctive, very small isolates were thought to belong to *Platyamoeba*, *Lingulamoeba* or *Clydonella* genera, but were unable to be distinguished on morphology alone.

Table 3.6: Species of *Paramoeba* isolated from fish with or at risk of amoebic gill disease

<i>Paramoeba</i>	Size	Isolation Method	Species
PA-002	> 25µm	Sea-water wash	<i>P. "pemaquidensis"</i> *
PA-011	45-100µm	Isopod trail	<i>P. eilhardi</i>
PA-012	> 25µm	Isopod trail	<i>P. "pemaquidensis"</i> *
PA-013	> 25µm	Isopod trail	<i>P. "pemaquidensis"</i> *
PA-014	> 25 µm	Isopod trail	<i>P. "pemaquidensis"</i> *
PA-016	> 25µm	Sea-water wash	<i>P. "pemaquidensis"</i> *

* morphology consistent with *P. pemaquidensis* (Page, 1983)

Table 3.7a: Morphology of amoeba isolates commonly found in the gills of fish during summer




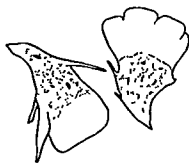

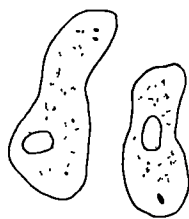
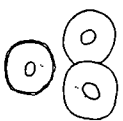
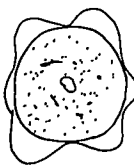
Genus	Locomotive form	Size	Inclusions	Nuclear Structure	Cysts	Floating Form	Flagellate Stage
<i>Acanthamoeba</i> 	Flattened. Subpseudopodia - slender, flexible and tapering from a broad hyaloplasm	12-15 μm	None	Central nucleolous	Outer layer wrinkled. Prominent endocyst wall 	Irregularly rounded with pointed pseudopodia 	None
<i>Flabellula sp.</i> 	Flattened, <i>Flabellulate</i> shape. No subpseudopodia from hyaline zone, though clefts may occur. Granuloplasmic mass preceded by extensive hyaloplasm often with uroidal filaments (Figure 3.9)	15-75 μm 8-30 μm	None	Some binucleate cells	None	Irregularly rounded without radiate pseudopodia 	None
<i>Heteroamoeba</i> 	Limax - "slug like" or cylindrical. No <i>Flabellulate</i> or spatulate forms. Eruptive locomotion (Figure 3.10)	12-47 μm	None	Nucleolar material parietal	Often with a delicate wrinkled outer membrane (Figure 3.11) 	Spherical with protruding rounded lobes 	2 long flagellate prominent anterior collar and anterior nucleus. Deep cytosome

Table 3.7b: Morphology of amoeba isolates commonly found in the gills of fish during summer

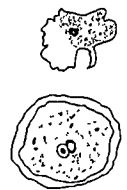

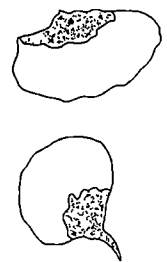


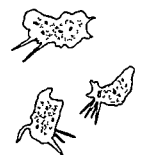
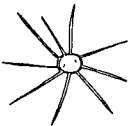
Genus	Locomotive form	Size	Inclusions	Nuclear Structure	Cysts	Floating Form	Flagellate stage
<i>Paramoeba</i> 	Compressed Subpseudopodia - produced from anterior hyaloplasm, tapering to rounded or fine sharp points (Figures 3.6 and 3.7)	45-100 μm (<i>P. eilhardi</i>) > 25 μm	1 or more DNA containing parasomes adjacent to the nucleus (Figures 3.6)	Usually larger than parasome Central nucleolus	None	Spherical with fine radiating subpseudopodia (Figure 3.8) 	None
<i>Platyamoeba/</i> <i>Vannella</i> 	Flattened, occasionally elongate. Granuloplasm preceded by flattened hyaloplasm. Hyaloplasm occupying anterior 1/3 to 1/2, often extending around the sides of the granuloplasm. Outline - oval, semicircular, elliptical, oblong, flabellate or spatulate	9-20 μm > 20 μm	None	Central nucleolus - most species Parietal nucleolar material - <i>Platyamoeba</i> <i>plurinucleolous</i>	None	Platyamoeba - radiate pseudopodia not tapering from base  Vannella - long, slender tapering pseudopodia tapering from the base 	None
<i>Vexillifera</i> 	Compressed. Subpseudopodia - slender conical projections from anterior hyaloplasm	Usually 5-20 μm	None observed	Central or parietal	None	Spherical with fine radiating pseudopodia 	None

Table 3.8: Distribution of amoebae genera between farms and sampling times

Date	Farm	Number of Fish Sampled	Number of Amoebae Isolated	Identity of Isolates
‘Wave’ 1				
28 October	C	11	-	
29 October	B	10	1	<i>Acanthamoeba</i>
12 November	A	12	4	<i>Platyamoeba/Vannella</i> (1)
27 November	C	10	-	
2 December	A	9	1	
3 December	B	10	1	<i>Paramoeba</i>
16 December	B	15	8	<i>Platyamoeba plurinucleolus</i> (2); <i>Small Platyamoeba</i> (2)
17 December	C	5	1	
19 December	A	10	6	<i>Platyamoeba/Vannella</i> (2)
‘Wave’ 2				
16 January	A	10	7	<i>Platyamoeba/Vannella</i> (4)
29 January	B	9	3	<i>Paramoeba</i> (2)
4 February	A	10	2	
14 February	C	12	17	<i>Flabellula</i> (7); <i>Platyamoeba/Vannella</i> (3)
‘Wave’ 3				
3 March	A	12	10	<i>Heteroamoeba</i> (1); <i>Paramoeba</i> (3); <i>Platyamoeba/Vannella</i> (1); <i>Vexillifera</i> (1)

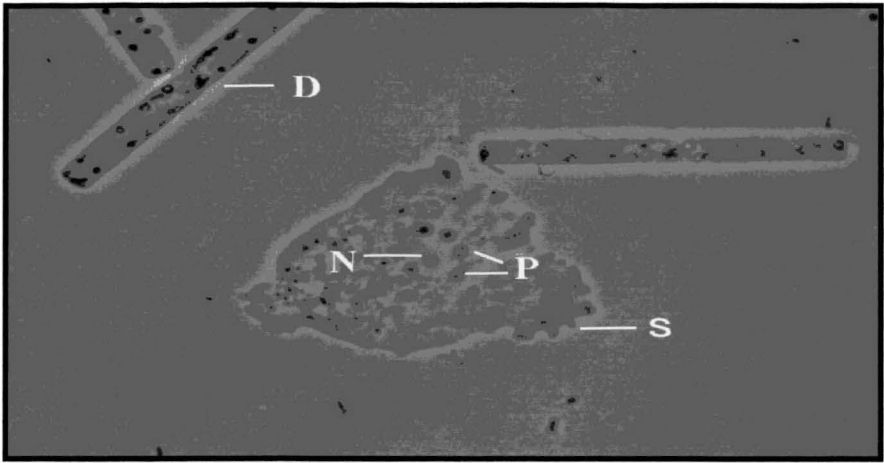


Figure 3.6: *Paramoeba* sp. ‘AGD’, showing 4 parasomes (P), nucleus (N), rounded subpseudopodia (S), and diatoms (D), phase contrast, X 1000

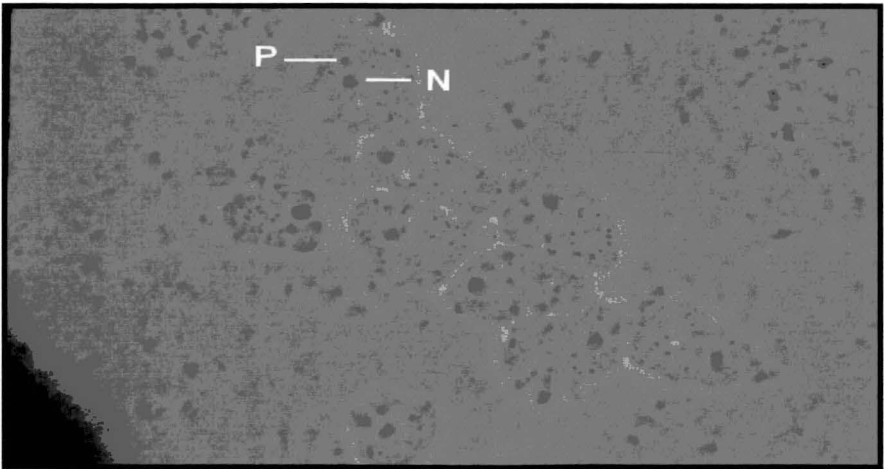


Figure 3.7: A cluster of *Paramoeba* sp. ‘AGD’, showing nucleus (N) and parasome (P), phase contrast, X 1000

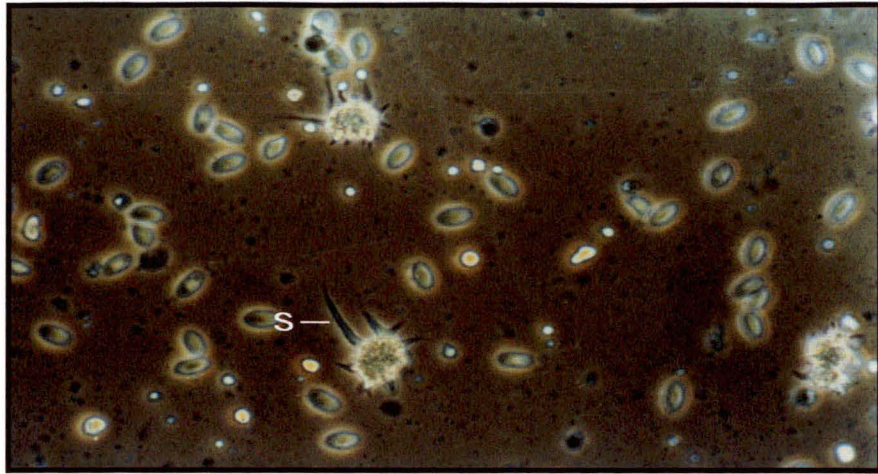


Figure 3.8: *Paramoeba* sp. 'AGD' floating form, showing fine subpseudopodia (S), light microscopy, X 400

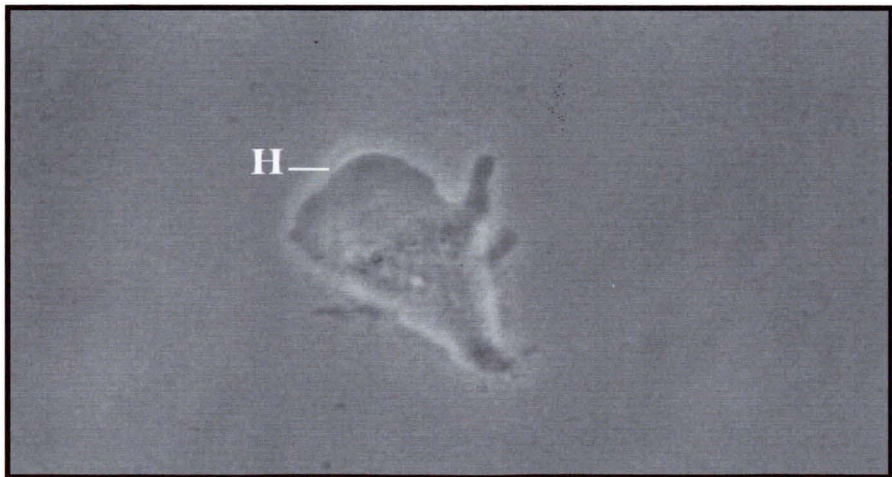


Figure 3.9: *Flabellula* species, showing bell-shaped hyaloplasm (H), phase contrast, X 1000

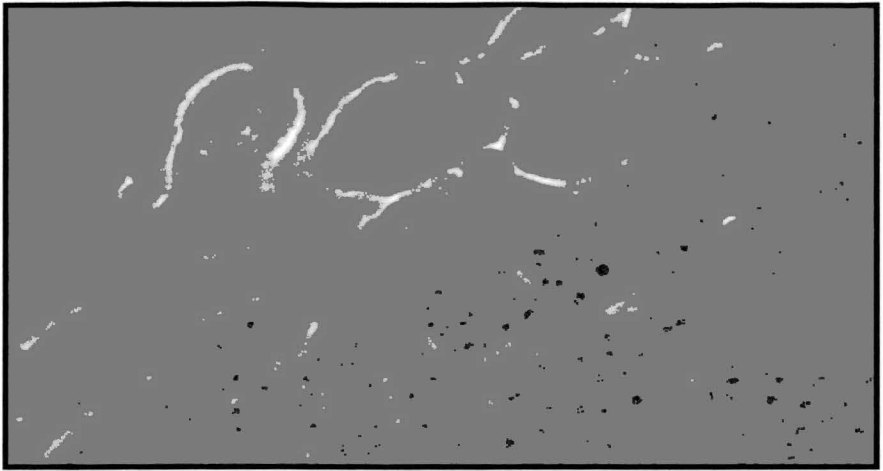


Figure 3.10: *Heteroamoeba* trophozoites, showing cylindrical ('limax') forms, phase contrast, X 1000

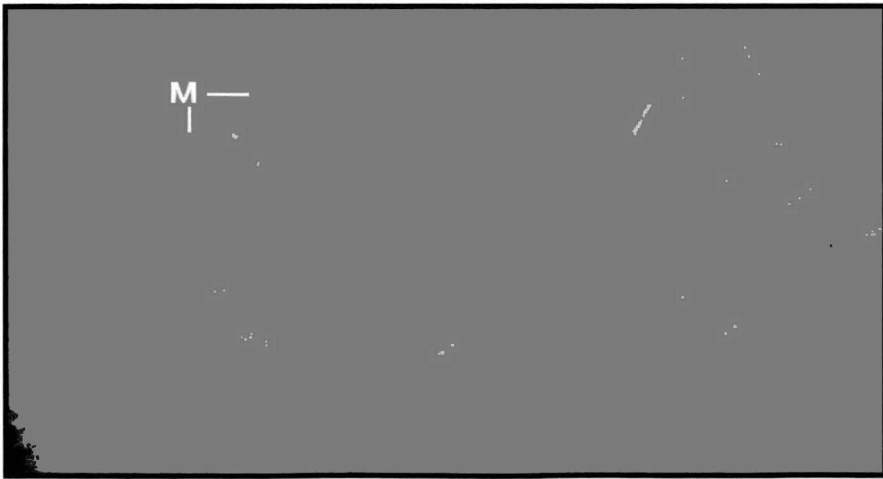


Figure 3.11: *Heteroamoeba* cysts, some with wrinkled outer membrane (M), phase contrast, X 1000

3.3.2 Verification of the pathogen

Gill health - histopathology: Isotonic Davidson's fixative was found to be superior to formalin in fixing amoebae in gill tissue. Formalin however gave better definition of gill damage. Both formalin and Davidson's fixed gill tissue were thus examined to give an overall picture of gill health.

Gills examined early in 'wave' 1 consisted of samples taken from fish 2, 12 and 30 days post acclimatisation. Little or no damage was observed in samples collected 2 to 12 days post acclimatisation (Figure 3.12). Fish that had been in the sea for 30 days showed some early responses to damage in the gills; slightly thicker tips of the primary lamellae, small numbers of lymphoid nodules, prominent chloride cells, early fusion of the secondary lamellae and an increase in mucus cells (Figure 3.13). No amoebae were observed in these sections.

Gills collected during the middle of 'wave' 1 showed a progression of the damage described above; multiple small and medium size lymphoid nodules, slight hyperplasia of the lamellae, increase in the number of chloride cells and an increase in mucus was observed. Larger areas of partial and total fusion of the secondary lamellae, pre-AGD lesions, were noted. A few amoebae were observed, in gill sections taken from one site.

Samples taken late in 'wave' 1 were variable and probably related to the environmental conditions present at the time of sampling. Pre-bathed samples from one site showed further progression of gill damage, with extensive AGD lesions characterised by large areas of fusion of the secondary lamellae and pseudostratification of the epithelial cells (formation of an extra layer of epithelial cells), and a further increase in mucus, and an increase in the number of mucous cells. Small numbers of amoebae were observed in some samples from this site. Post bathed fish from the same site showed similar gill pathology but with less mucus, and no amoebae. Osmotic changes were observed in samples taken from the remaining sites; swollen secondary lamellae and secondary epithelial cells, plump mucous cells and increased mucus production. Both of these sites had experienced unseasonably heavy rain during the sampling period, resulting in a layer of freshwater 5-6 feet deep on top of the sea-water.

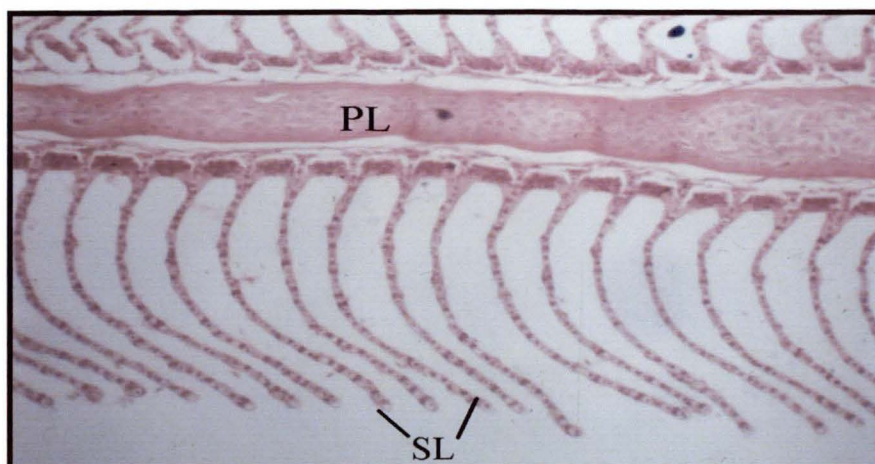


Figure 3.12: Histopathology of normal Atlantic salmon gill tissue, showing primary (PL) and secondary lamellae (SL). H&E stained, X200



Figure 3.13: Early responses to gill damage showing an increase in mucous cells (mc) and slightly thickened tips of the secondary lamellae. H&E stained, X200

Subsequent samples taken late in 'wave' 2 showed a similar progression of gill damage; an increase in the number and size of lymphoid nodules, larger areas of partial fusion of the secondary lamellae, an increase in the number and size of AGD lesions, more pseudostratification and increased mucus stringing between the lamellae (Figures 3.14 and 3.15). Amoebae were observed in these sections but were still relatively few in number. Post-bathed samples had similar pathological changes without amoebae and with little or no mucous strings.

Fish sampled during 'wave' 3 showed a higher frequency of well developed AGD lesions and pseudostratification. A small number of amoebae were observed associated with lesions. Figures 3.16 to 3.18 show examples of typical AGD lesions, while Figure 3.19 shows severe AGD with few amoebae; these sections were not from fish sampled during this study and were taken subsequently from AGD infected fish to illustrate the progression of disease.

Examination of isopod sections showed a variety of associated organisms; large ciliates; bacillary bacteria, some filamentous bacteria; fungi; diatoms and possibly spirochaetes. Red blood cells of fish origin were observed in crevices adjacent to the mandibles in some sections. Intracellular inclusions, resembling virus particles, were also noted. No amoebae were observed in these sections.

Antiserum: Antisera to the four amoeba isolates were highly specific. The anti-sera only reacted to the amoeba isolate from which they were prepared. Antiserum made to *Paramoeba* (PA-016) reacted positively with all five *Paramoeba* 'AGD' isolates; but did not react with any of the 42 other amoeba isolates tested. As the *P. eilhardi* isolate was unable to be maintained in culture the extent of any cross reactivity could not be determined.

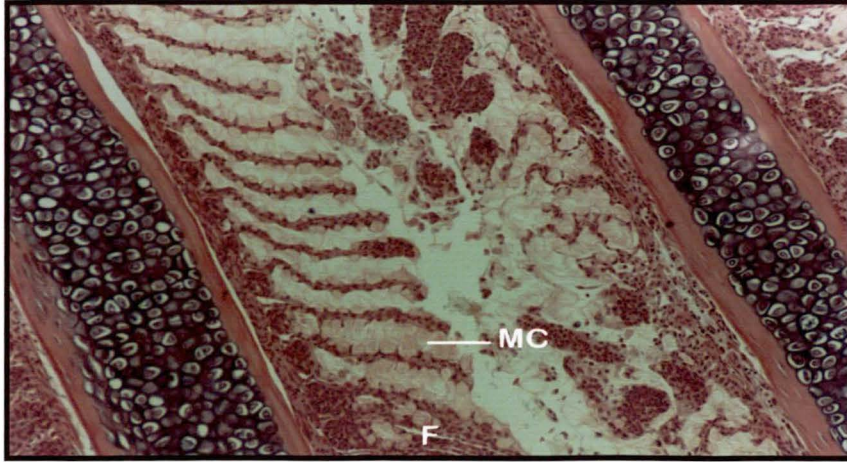


Figure 3.14: Progression of gill damage showing areas of partial fusion of secondary lamellae (F), increase in mucous cell number (MC). H&E stained, X200.

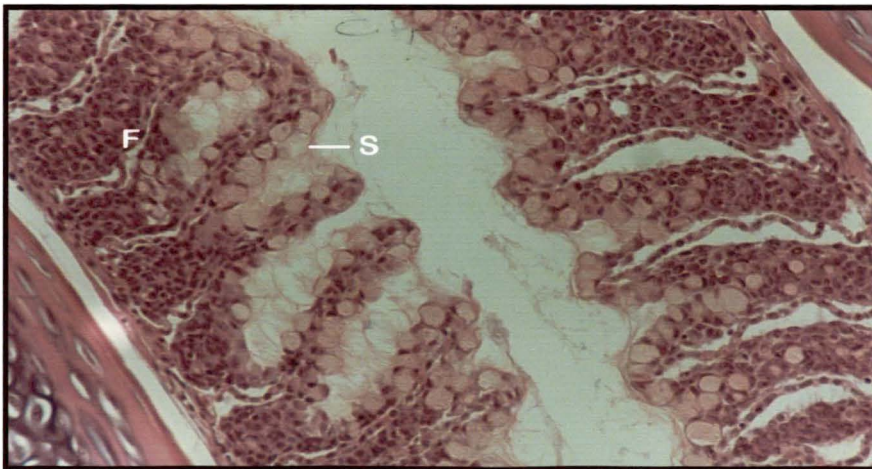


Figure 3.15: Close-up of Figure 3.14, showing fusion of secondary lamellae (F), mucous cells and mucus stringing between the lamellae (S). H&E stained, X400.

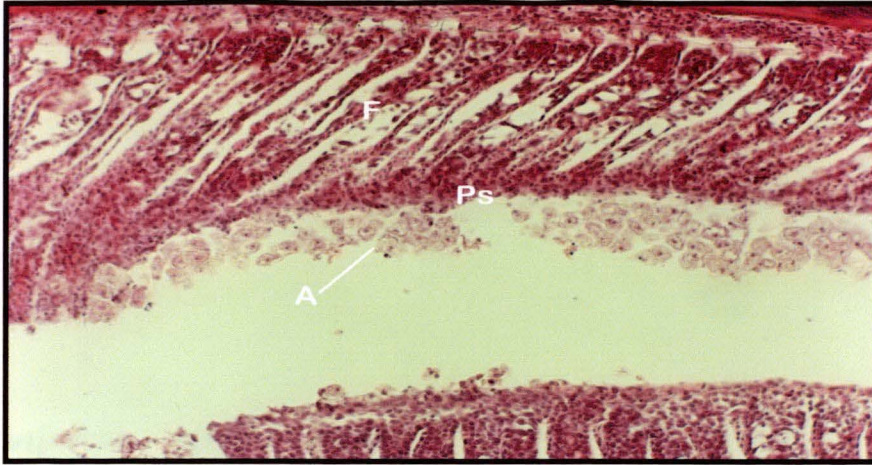


Figure 3.16: Typical AGD affected gill with extensive fusion of the secondary lamellae (F), pseudostratified epithelium (Ps) and numerous amoebae (A). H&E stained, X200.

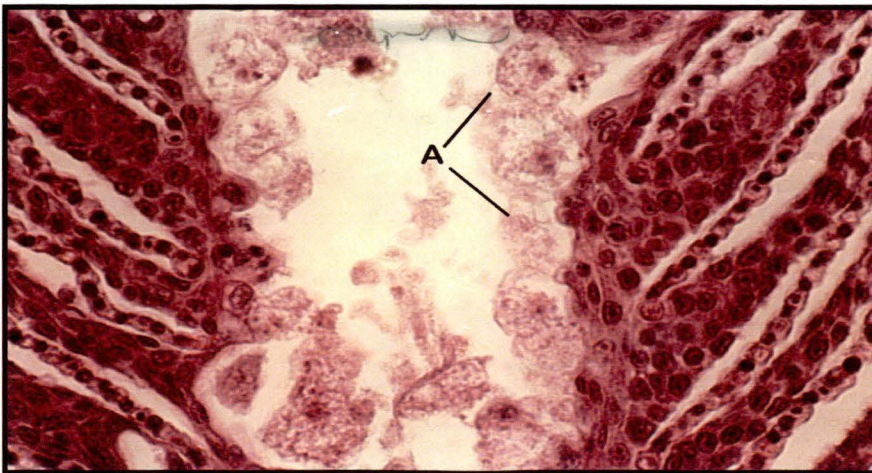


Figure 3.17: Close-up of AGD affected gill showing fusion of secondary lamellae and the presence of amoebae (A) adhered to the damaged tissue. H&E stained, X400.

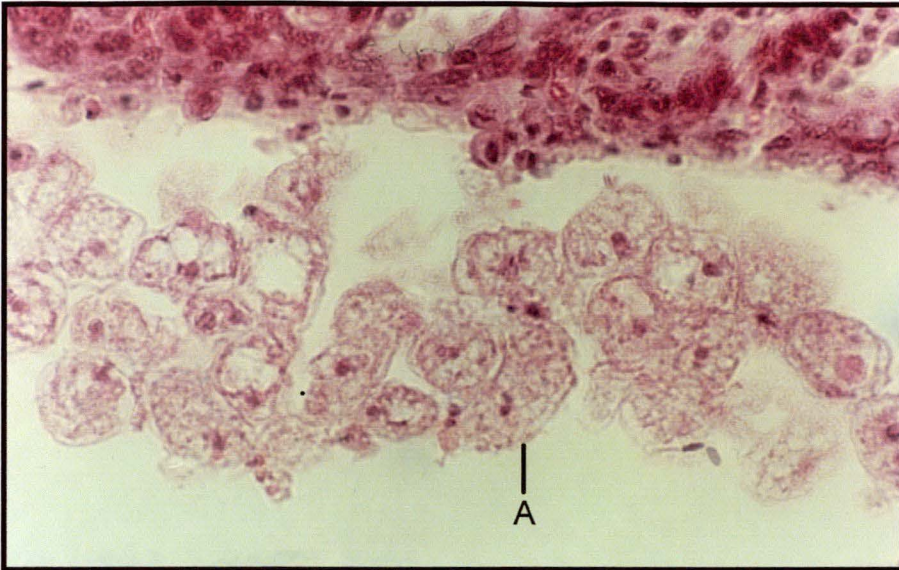


Figure 3.18: AGD affected gill with numerous amoebae (A). H&E stained, X400.

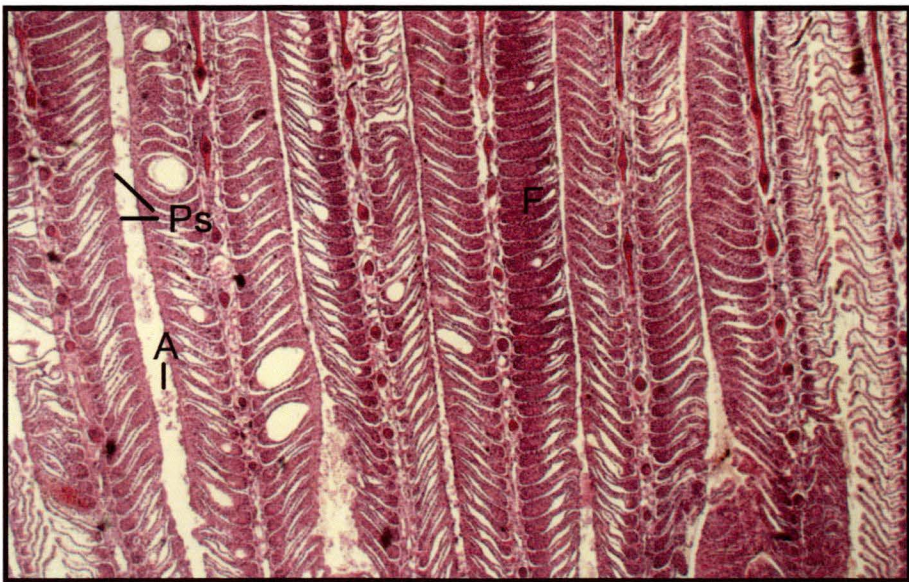


Figure 3.19: Severe AGD infected gill showing extensive areas of fusion (F) and pseudostratification of the epithelial cells (Ps), with few amoebae (A) in the spaces between the primary lamellae. H&E stained, X40.

Immunostaining

Immunofluorescence: Table 3.9 shows the results of immunofluorescence staining of gill sections known to be positive for amoebae by H&E staining, with the four antisera prepared. Nearly all sections contained fluorescent labelled amoebae when tested with *Paramoeba* antiserum (36/37); few sections were positive when tested with *Flabellula* antiserum (3/29), *Vannella/Platyamoeba* antiserum (2/32), and *P. plurinucleolus* antiserum (5/32). Large numbers of fluorescent amoebae were observed in many of the sections stained with *Paramoeba* antiserum (Figures 3.20 – 3.21), some encrypted within the damaged gill tissue (Figure 3.22). The number of fluorescent amoebae or amoeba-like bodies was very small in sections immunostained with the other three antisera, on average between 1-3 amoebae were observed per section. In some of these sections it was difficult to determine if the fluorescence was due to artefact. Sections from two isopods were screened with the four antisera; amoebae were detected with antisera to *Paramoeba* (3 amoebae), *Vannella/Platyamoeba* (3), *P. plurinucleolus* (1) and *Flabellula* (1). Protease digestion did not enhance the immunofluorescence staining.

The immunofluorescence technique was more sensitive than H&E staining detecting amoebae in 19/22 gill tissue samples, compared to only 7/22 by examination of H&E stained gill tissue. Large numbers of *Paramoeba* were observed in all five gill samples collected from harvest sized fish affected with AGD in winter. Fluorescent labelled amoebae were observed in all gill sections sent from New Zealand and France immunostained with *Paramoeba* antiserum.

Large numbers of amoebae were observed in frozen sections immunostained with *Paramoeba* antiserum from farm A, collected late in May 1992. Fewer amoebae were noted in samples taken from farm B in December of 1991. In frozen gill material, many of the amoebae were not attached to the damaged gill tissue, whereas in chemically fixed material there were fewer free amoebae seen, most being in close association with the gill. An isopod collected from farm A at the same time as the gill samples showed a moderate number of amoebae, not attached to the exoskeleton of the isopod.

Table 3.9: Results of immunofluorescence staining of gill sections, known to be positive for amoebae by histological examination, with antisera to the four amoeba isolates

Fixed Sections	<i>Antisera</i>			
	<i>Flabellula</i> sp.	<i>Platyamoeba/Vannella</i> sp.	<i>Platyamoeba plurinucleolus</i>	<i>Paramoeba</i> sp.
1991/92 season				
‘Wave’ 1	1/9*	1/9*	2/9*	9/9
‘Wave’ 2	1/7*	1/7*	1/7*	7/7
‘Wave’ 3	0/4	0/4	0/4	3/4
Winter 1992	1/5*	0/5	1/5*	5/5
<i>Previous Years</i>				
1985-91	0/4	0/7	1/6*	12/12
Total	3/29	2/32	5/31	36/37

* represents 1-3 fluorescent bodies detected per section
Numerator = number of sections positive for amoebae by immunostaining
Denominator = number of sections positive for amoebae by histological examination

Immunoperoxidase: Selected sections found positive by immunofluorescence staining were tested by the immunoperoxidase method, in order to observe the association of amoebae with the gills of AGD affected fish. Only *Paramoeba* antiserum was tested due to the large number of positively staining amoebae by immunofluorescence. *Paramoeba* were observed in large numbers associated with damage in some sections, often lining the damaged lamellae (Figures 3.23 and 3.24). Comparison of H&E stained sections with immunoperoxidase stained sections demonstrated that most, if not all, the amoebae associated with AGD related tissue damage were *Paramoeba* species (Figures 3.25 and 3.26).

Osmium tetroxide intensified the peroxidase reaction but resulted in a dark background that masked the counterstain. A greater contrast was achieved between the gill tissue and the amoebae using the methyl green as a counterstain, however it resulted in a greater damage to the gill tissue. Alkaline phosphatase staining provided a better contrast between gill and *Paramoeba* (Figures 3.27 and 3.28).

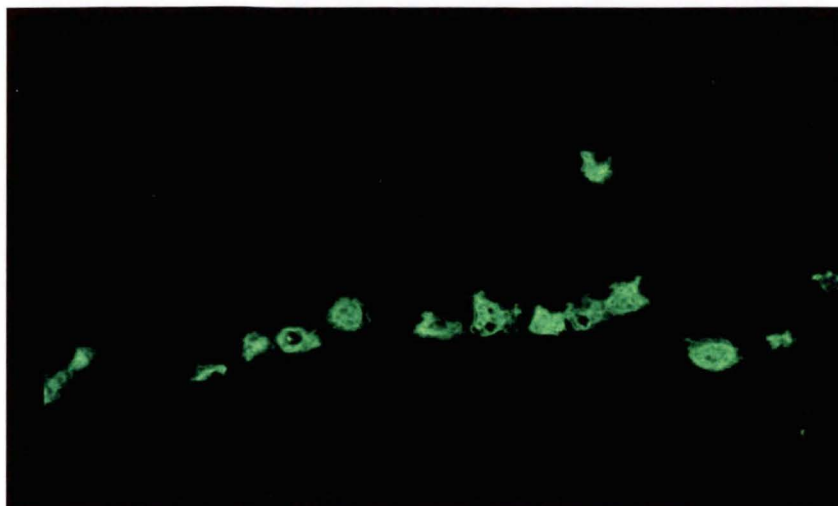


Figure 3.20: Immunofluorescent stained gill using *Paramoeba* antiserum, showing the presence of fluorescent *Paramoeba* lining the pseudostratified epithelium (not visible). X100

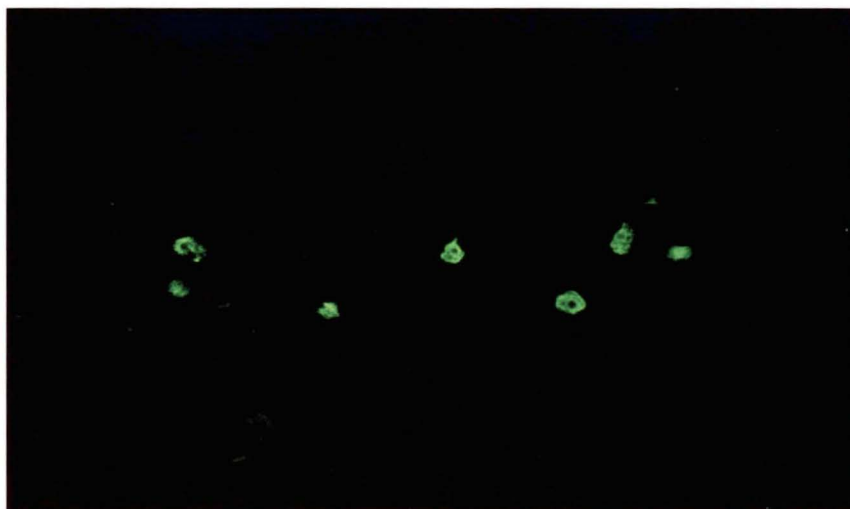


Figure 3.21: Immunofluorescent stained gill using *Paramoeba* antiserum, showing the presence of fluorescent *Paramoeba* in and attached to the gill tissue (barely visible). X100

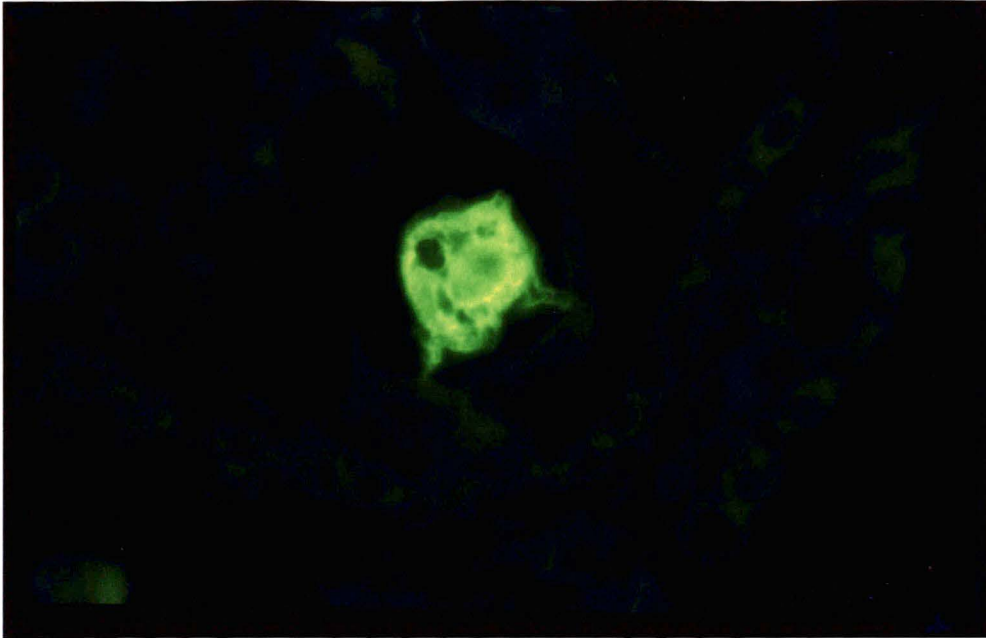


Figure 3.22: Immunofluorescent stained gill using *Paramoeba* antiserum, showing the presence of a fluorescent *Paramoeba* encrypted within the damaged gill.
X400

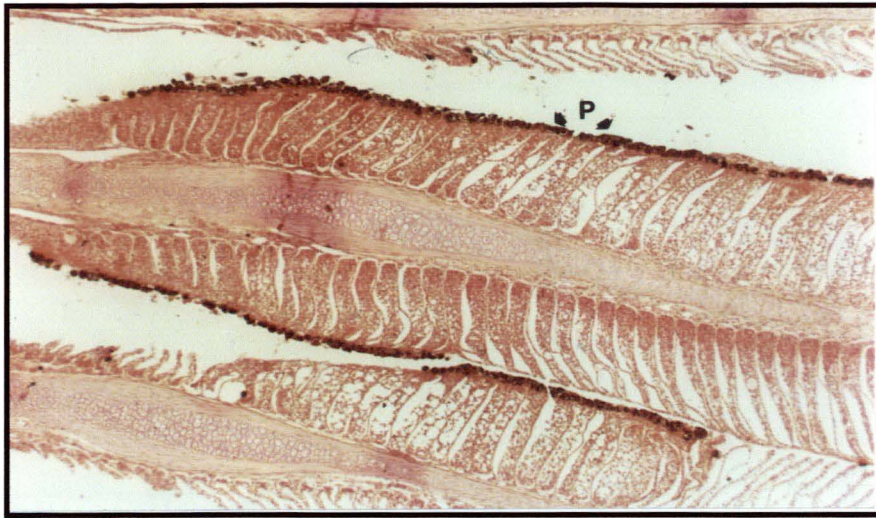


Figure 3.23: AGD affected gill immunoperoxidase stained with *Paramoeba* antiserum, showing stained *Paramoeba* (P) lining the damaged tissue. X100

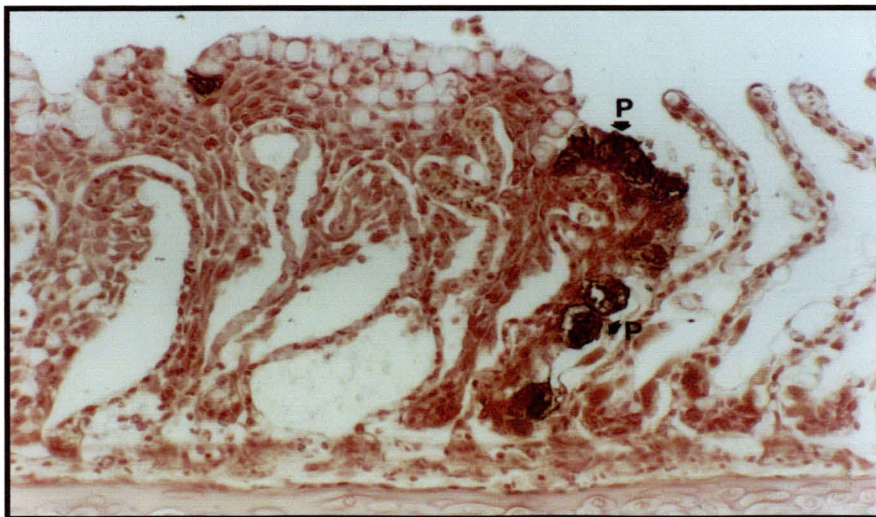


Figure 3.24: AGD affected gill immunoperoxidase stained with *Paramoeba* antiserum, showing stained *Paramoeba* (P) in close association with an area of fused secondary lamellae. X200

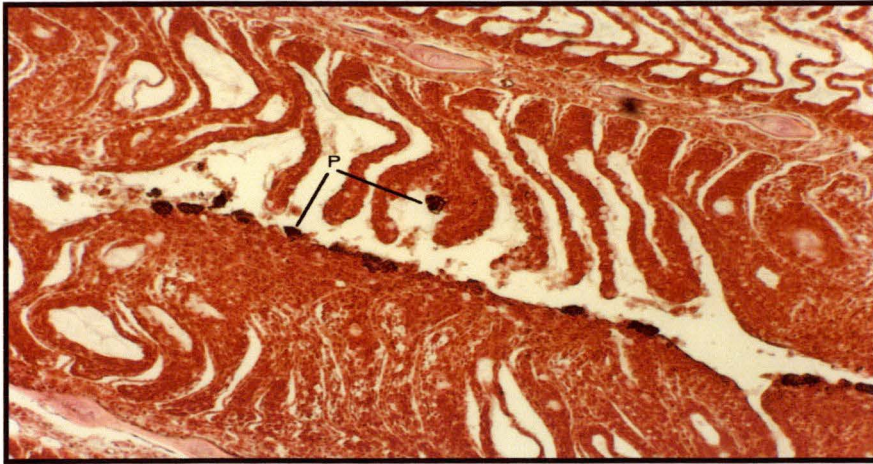


Figure 3.25: AGD affected gill immunoperoxidase stained with *Paramoeba* antiserum, showing a small number of stained *Paramoeba* (P) attached to the damaged tissue. X100

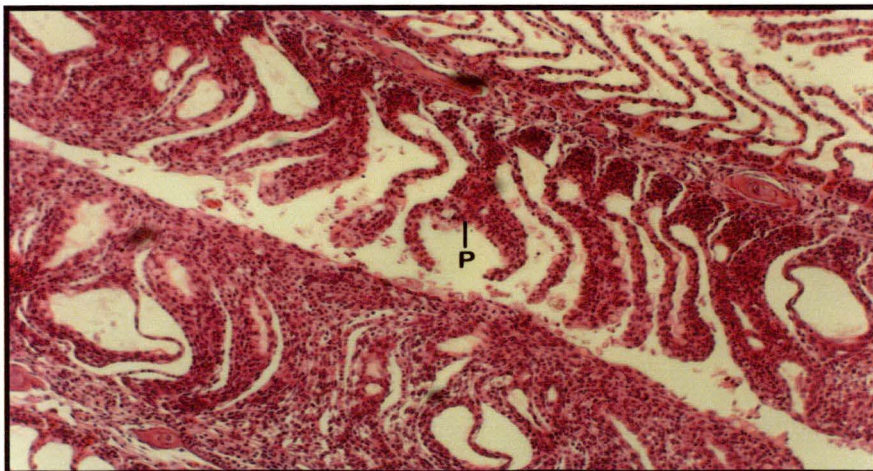


Figure 3.26: Section taken from the same tissue block as Figure 3.25, H&E stained, showing few discernable *Paramoeba* (P). X100

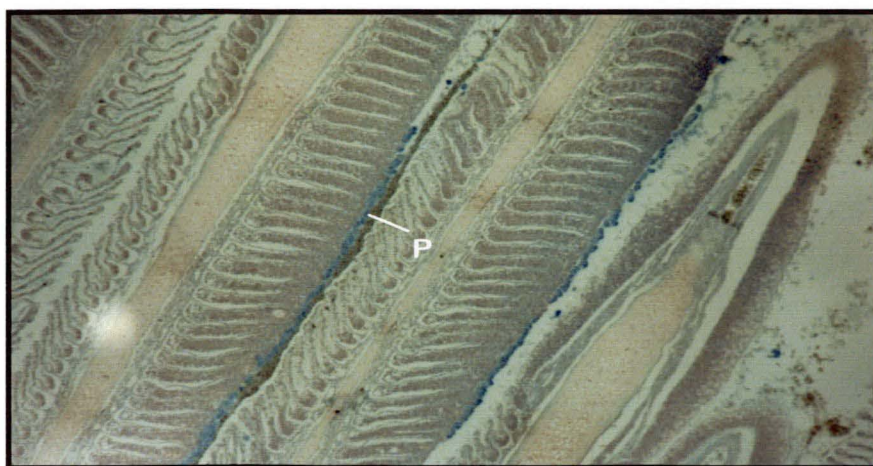


Figure 3.27 :AGD affected gill tissue immunoalkaline phosphatase stained with *Paramoeba* antiserum, showing *Paramoeba* (P) lining the damaged gill, and demonstrating greater contrast with the gill tissue. X100

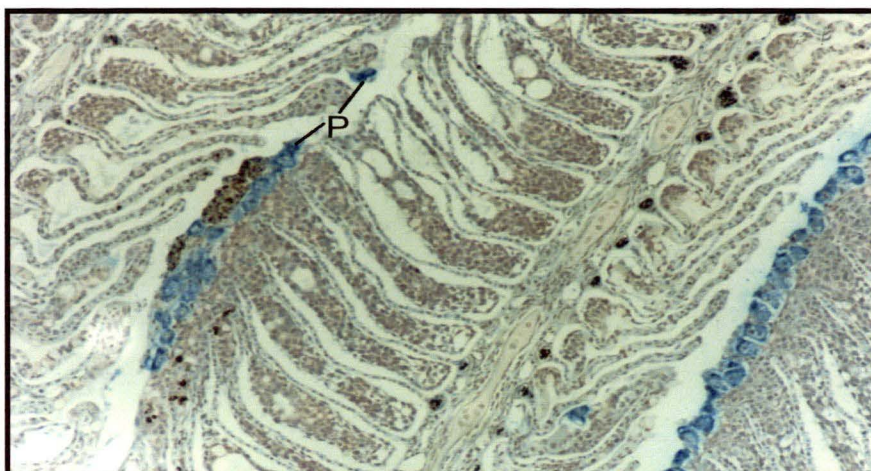


Figure 3.28: Close-up of Figure 3.27, showing the enhanced contrast between the gill and the stained *Paramoeba* (P), and the close association of the amoebae with the gill tissue. X200

3.3.3 Additional Investigations

Long term preservation: Samples of the *Platyamoeba plurinucleolus* isolate were successfully revived from freezing at -70°C over a 6 month period. No attempt was made to quantitate how many amoebae survived the freezing technique. There were no apparent differences observed between the amoebae frozen in sea-water and those frozen in sea-water containing DMSO.

Most isolates frozen with the cryopreservation medium survived the freezing and retrieval process when examined after 2-3 weeks at -70°C. In this study the range of amoebae isolated from the gills of fish with AGD and able to be preserved with this method encompassed isolates from the genera, *Acanthamoeba*, *Flabellula*, *Heteroamoeba*, *Vexillifera*, *Vannella*, and *Platyamoeba*. Of the amoeba isolates not classified into genera, at least five did not survive the process. *Acanthamoeba* and *Heteroamoeba* species preferred culture on wet plates after retrieval, for the cysts to excyst. *Flabellula* species also preferred wet plates for optimum growth after freezing. Isolates successfully preserved using this method were periodically retrieved over a three and a half year period, resulting in viable cultures.

Despite all attempts none of the *Paramoeba* isolates were able to be successfully cryopreserved. Microscopic examination of the cells immediately after retrieval showed rounded translucent cells that appeared to be intact, with no growth being recorded.

Refining isolation technique: Immunofluorescence testing of ammonium chloride extracted gill material with anti-*Paramoeba* antibody showed the presence of large numbers of specifically stained amoebae in 2/3 fish sampled. Many were observed trapped in the mucus. One fish showed very few specifically staining amoebae.

Fourteen amoeba isolates were grown from the ammonium chloride wash, five of these being *Paramoeba* species. Four morphologically distinct amoebae were isolated from one fish, one isolate was cyst-forming, but none were *Paramoeba* sp. Nine amoeba isolates were grown from a second fish, of which four were *Paramoeba*, and one was cyst-forming. One *Paramoeba* isolate was cultured from the third fish sampled. All *Paramoeba* isolates were identified as *Paramoeba* species due to the presence of one or more parasomes, identified using by phase contrast microscopic examination of the isolates immobilised onto agar coated slides (Method 2.3.1) and confirmed by the DAPI technique (Method 2.3.2).

Confirmation of the parasome: The DAPI technique was extremely successful in identifying the presence of DNA containing bodies such as nuclei and parasomes. This technique allowed the morphology of the nuclei to be distinguished clearly, and in the case of *Paramoeba* species allowed the parasomes to be clearly distinguished. Phase contrast microscopy of amoebae adhering to agar slides was also successful in showing the presence of parasomes, however, this technique was more time consuming, more time being required to find suitably flattened amoebae for observation. The Gomori's trichrome staining technique was also successful in identifying more than one DNA containing body, however the process resulted in dehydration of the cells making identification a little difficult to distinguish from artefact.

Preliminary identification of *Paramoeba* isolates: The growth characteristics of amoeba isolates was sufficiently different in some genera to allow preliminary identification of isolates based on this characteristic alone. Examination of the growing characteristics of isolates allowed many amoebae to be eliminated as belonging to the genus *Paramoeba* and an almost always resulted in the identification of *Paramoeba* sp. 'AGD'. Table 3.10 describes the growth characteristics *Paramoeba* sp. 'AGD' isolated from the gills of Atlantic salmon, compared with other commonly isolated amoebae. (Also see Figures 3.29 – 3.38)

Table 3.10: Growth characteristics of *Paramoeba* sp. ‘AGD’ isolated from the gills of Atlantic salmon: compared with other commonly isolated amoebae.

Amoebae	Growth Front Characteristics	Older Growth Characteristics
<i>Paramoeba</i> species (associated with AGD)	In healthy cultures growth front forms a thick “wall” of cells. Some single cells move ahead of the growth front, which appears slightly more raised (Figures 3.29 + 3.30). In less healthy cultures or cultures with limited bacterial substrate the “wall” may be less defined (Figure 3.31).	Amoebae often occur in distinct clusters or “rosettes” (Figures 3.32 + 3.33). Amoebae generally flattened.
<i>Flabellula</i> species (isolated from gills)	Growth front usually a thick sheet of cells, made up of large numbers of cells, honeycomb in appearance, some cells observed ahead of the front (Figure 3.34).	Amoebae occur in large raised clusters, with a similar honeycomb appearance (Figure 3.35).
<i>Platyamoeba</i> and <i>Vannella</i> species (isolated from gills)	Most species isolated from a distinct growing front, which is usually thin and raised, with no cells ahead of the growth front (Figures 3.36 + 3.37).	Amoebae occur singly, no clusters observed (Figure 3.38).
Euryhaline species, <i>Acanthamoeba</i> and <i>Heteroamoeba</i> . (isolated from gills)	Growth front variable, sometimes thick. Amoebae are raised in <i>Acanthamoeba</i> species and flattened in <i>Heteroamoeba</i> species isolated.	No amoebae trophozoites present in areas of older growth only cysts, which appear grain like and are highly refractive.

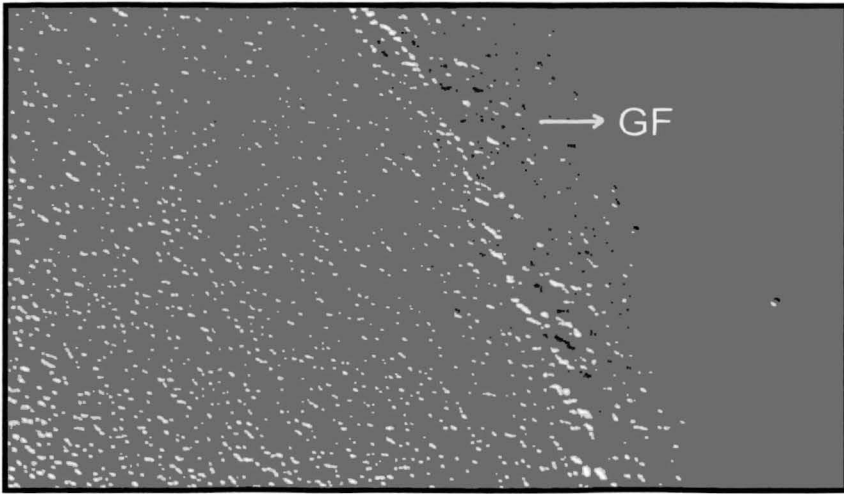


Figure 3.29: Healthy *Paramoeba* ‘AGD’ sp. growth front (GF), X 35

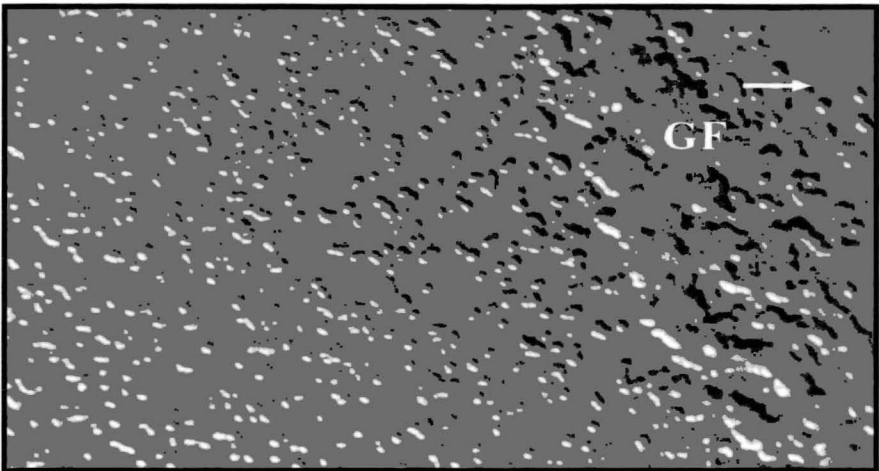


Figure 3.30: Healthy *Paramoeba* sp. ‘AGD’ growth front (GF), X 70

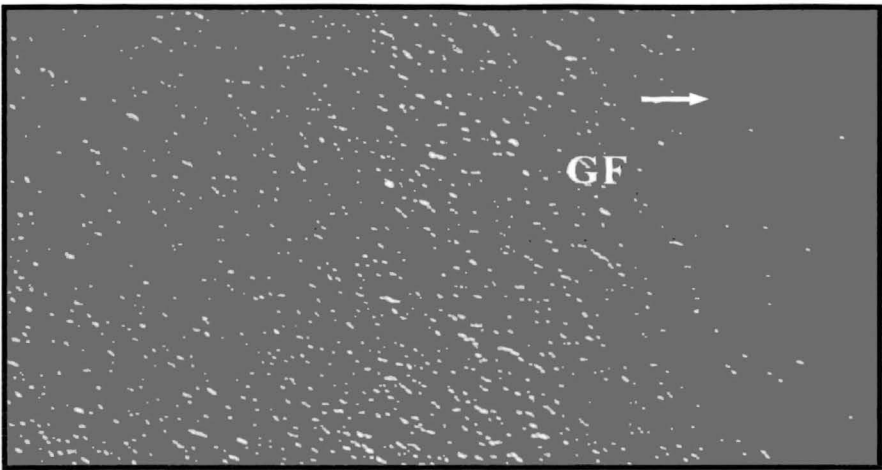


Figure 3.31: Less healthy *Paramoeba* sp. ‘AGD’ growth front (GF). Note diffuse growth front, X 35

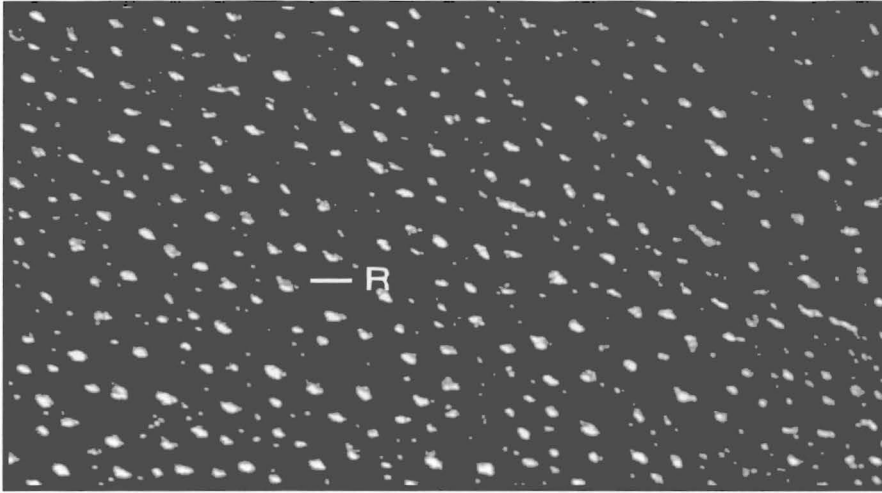


Figure 3.32: Older growth *Paramoeba* sp. ‘AGD’ forming ‘rosettes’ (R), X 35

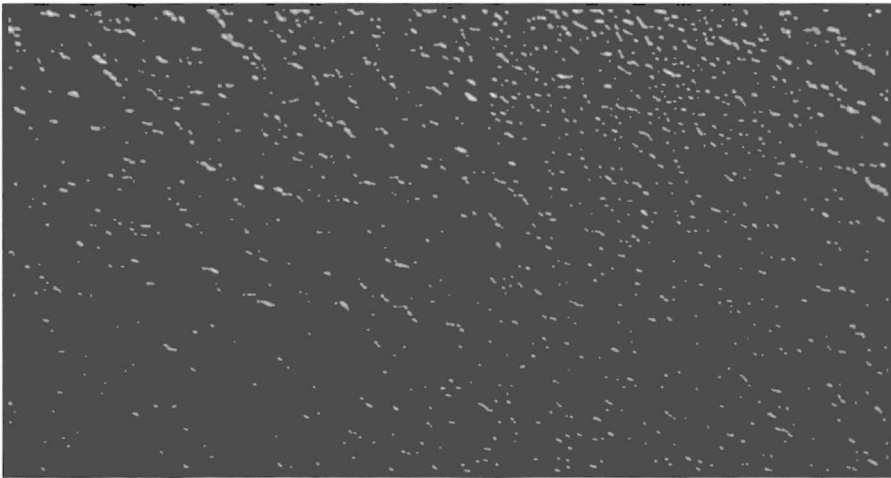


Figure 3.33: Older growth *Paramoeba* sp. ‘AGD’, showing variable clusters, X 35

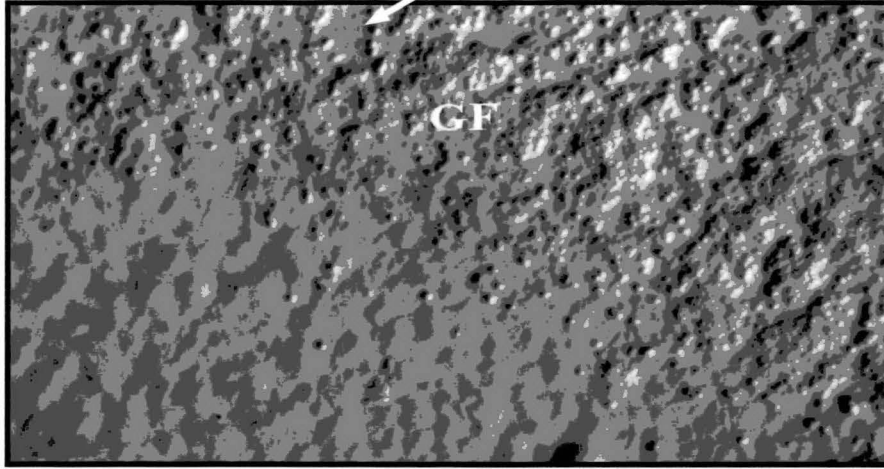


Figure 3.34: *Flabellula* sp. growth front (GF), showing sheets of raised cells, X 35

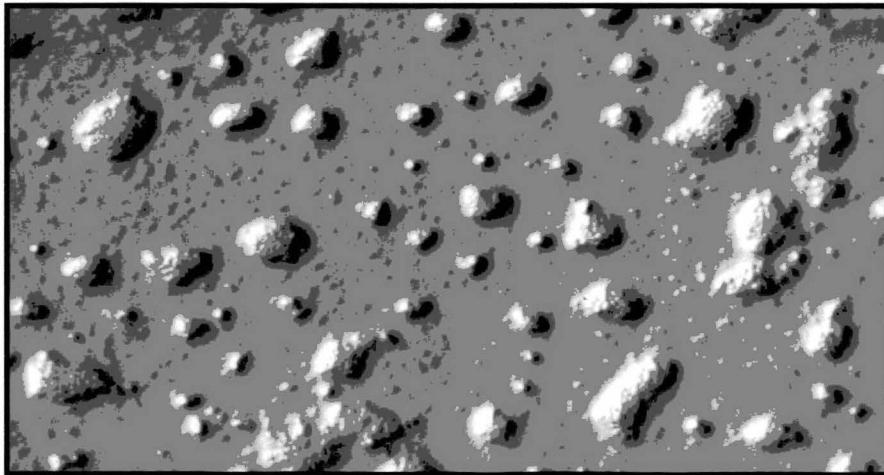


Figure 3.35: Older growth *Flabellula* sp., showing raised clusters of cells, X 70

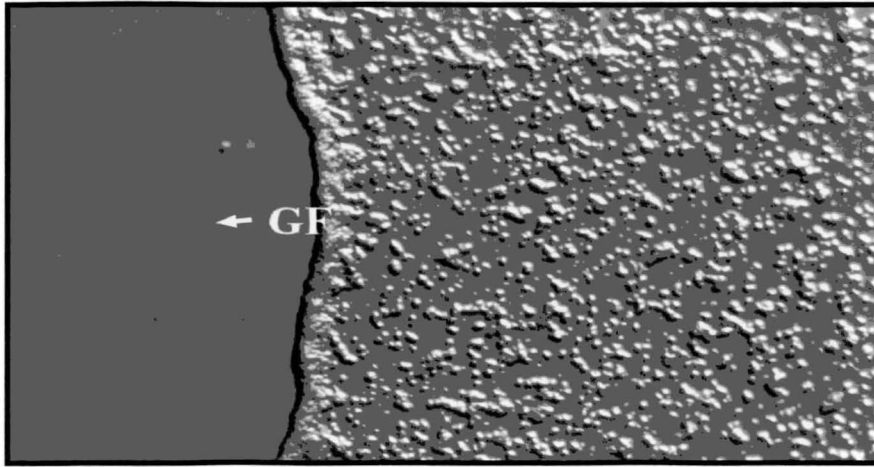


Figure 3.36: *Platyamoeba/Vannella* sp. growth front (GF), showing a distinct wall of cells, X 35

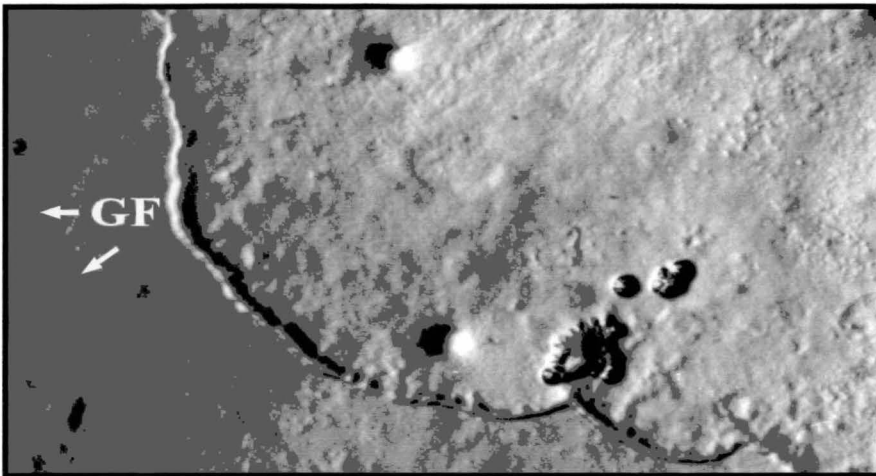


Figure 3.37: *Platyamoeba/Vannella* sp. growth front (GF), small species, individual cells not clearly visible, X 35

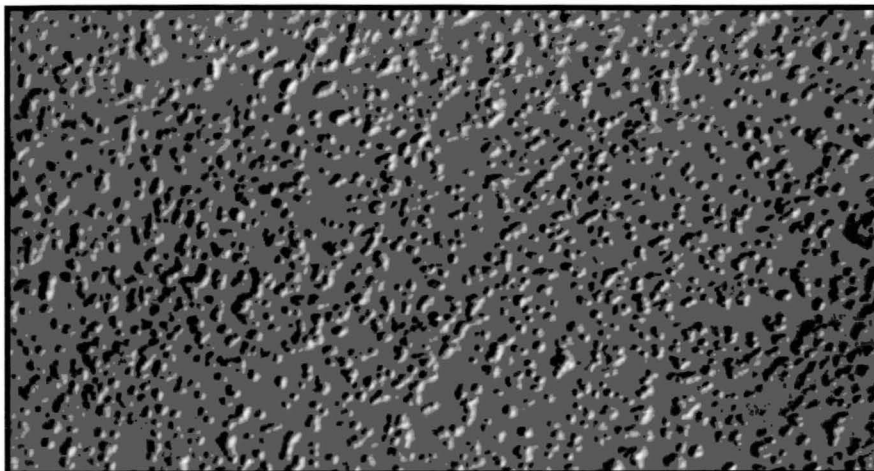


Figure 3.38: Older growth *Platyamoeba/Vannella* sp., showing single cells, no clusters, X 35

3.4. DISCUSSION

3.4.1 Isolation and Characterisation

It is important to establish which genera or species of amoebae are involved in AGD. Little importance had been attached to the isolation and identification of amoebae, other than *Paramoeba*, found in the gills of AGD infected fish. This study has shown that the majority of isolates cultured from the gills of fish with and at risk of AGD, were not *Paramoeba*. At least seven genera were found present in the gills of fish with and at risk of AGD: *Platyamoeba*, *Vannella*, *Heteroamoeba*, *Acanthamoeba*, *Flabellula*, *Vexillifera* and *Paramoeba*. These findings explain the anomalies observed in isolates collected in previous years, and later found not to be *Paramoeba* sp. (P. Larson and P. Statham, Pers. Comm.). The absence of a fully characterised and validated pathogen/s has made it difficult to undertake with any certainty, *in vitro* drug trials, pathogenicity trials, or to develop diagnostic tests to detect pathogens in clinical material.

In Tasmania, isolation of amoebae from fish with and at risk of AGD has been attempted by a number of researchers in previous years (Jones, 1988; P. Statham, Pers. Comm.). Various loosely defined methods have been used to isolate amoebae, but their effectiveness had not been clearly defined. Kent *et al* (1988) isolated *P. pemaquidensis* from coho salmon in the USA by placing infested gill tissue into a complex tissue culture medium. Attempts to isolate and maintain amoebae using this method in our laboratory have been unsuccessful (work not included in the thesis). Isolation methods can be, by nature, selective and thus, in the absence of a proven method, a number of procedures were employed to maximise the likelihood of isolating all amoebae associated with AGD. In addition the sampling was undertaken at three different sites, two fully marine sites with typical AGD progression and one site subject to freshwater flushes (a phenomenon that acts as a natural treatment for AGD, sometimes causing an atypical progression of disease).

Five methods were employed and a total of 680 gill cultures were prepared and examined during this study, resulting in 61 cloned amoeba isolates. The most successful method in isolating amoebae was the sea-water wash method, which recovered 21 isolates. The success of this method was most likely due to the prolonged agitation of the gills in sea-water containing antibiotics, leading to gill concentrates being rendered relatively free of contaminating bacteria. Amoebae if present were then able to grow without competition. Bacteria present as normal flora in the gills presented a difficulty when attempting to isolate amoebae, as bacterial overgrowth tended to mask or inhibit the growth of amoebae. Despite using a non-nutrient agar and antibiotics in the agar plates, bacteria were still observed to overgrow the amoebae, especially in the gill tissue method. Antibiotics in the agar alone were not sufficient to inhibit bacterial growth in this method, as not all the gill surface was in contact with the agar. This may explain why this method was not as successful as the sea-water wash method. The freshwater wash method was employed to mimic the mechanical stripping of mucus and amoebae, a process believed to occur during freshwater bathing. Although gill mucus could be seen macroscopically in sample preparations, recovery rates of amoebae by this method were low. The low recovery rate of this method may be due to freshwater being lethal to the amoebae present on the gills of fish with AGD, a suggested therapeutic effect of freshwater bathing (Jones, 1988; Munday *et al*, 1990). It is not known why the sea-water well method was not very successful at isolating amoebae, since bacterial overgrowth was slight in this method.

A significant number of amoebae were isolated from isopod trails. Isopod trails were not heavily contaminated with bacteria or other contaminating organisms, which may explain the high frequency of isolation. Success using this method suggests that amoebae are present on, or associated with, the isopods. The role of isopods in AGD is not clear, and isolation of amoebae from isopods suggests they may act as a reservoir or as a vector/transport host.

Most of the amoeba isolates were cultured from material collected late in the waves of infection, when signs of AGD were present. Very few amoebae were isolated early or midway through 'wave' 1, indicating that amoebae were not present at significant levels or that they were not present at levels high enough to be detected using the methods described.

Amoebae belonging to the genera *Platyamoeba* and *Vannella* were the most commonly isolated marine amoeba in this study, which is not unusual given that *Platyamoeba* species have been reported as the most commonly isolated marine genus (Page, 1983). Species of amoebae belonging to the genera *Platyamoeba* and *Vannella* have been reported from turbot with AGD (Leiro *et al*, 1998; Dyková *et al*, 1999). A *Platyamoeba* species was first thought to be the agent of AGD in turbot (Leiro *et al*, 1998), but later a *Paramoeba* species was found to be the causative agent (Dyková *et al*, 1998c). The *Platyamoeba/Vannella* isolates cultured in this study represented a range of distinct morphological types and sizes, which is supported by Dyková *et al* (1999) where the *Platyamoeba* species isolated from turbot represented a range of sizes. *Vannella platypodia* has been isolated from the kidney tissue of the common goldfish (*Carassius auratus*) and from the brain of chub (*Leuciscus cephalus*), but were not associated with abnormal pathology or disease. Both of these fish species being cultured in freshwater (Dyková *et al*, 1996; Dyková *et al*, 1998a). It is unlikely that any of the *Platyamoeba/Vannella* isolates cultured in this study were *Vannella platypodia*. There are no reports of *Vannella* or *Platyamoeba* species as pathogens in marine or freshwater hosts.

Flabellula calkinsi has been isolated from the digestive tract of oysters, with no evidence of pathogenicity, and free-living isolates have also been described (Page, 1983). All other *Flabellula* species described are free-living (Page, 1983). The *Flabellula* species isolated in this study were morphologically similar; approximately 20-30µm in size, with uroidal filaments and some binucleate cells, consistent with either *F. calkinsi* or *F. citata* as described by Page (1983). They were only cultured from farm C at one sampling time. The occurrence of this species at this site may be the direct result of the presence of an oyster farm adjacent to the site, the oysters

acting as a reservoir for these organisms. Examination of these isolates by electron microscopy was not performed; if performed the unique surface structures of *F. calkinsi* and *F. citata* may have allowed us to eliminate or differentiate between these two species. *Flabellula* species have also been isolated from turbot with AGD, the dimensions fitting the range given by Page (1983) for *F. calkinsi* and *F. citata* (Dyková *et al*, 1999).

The genus *Heteroamoeba* contains a single marine species that commonly occurs in relatively brackish marine environments (Page, 1983). The *Heteroamoeba* sp. isolated in this study was cultured from farm A, which is subject to freshwater flushes, which may explain its occurrence there. Its isolation was not considered significant in this study.

Acanthamoeba species are freshwater or euryhaline amoebae commonly occurring in freshwater and soil; they are able to tolerate extreme environmental conditions, at least as cysts, in waters of varying salinities (Page, 1983). Its occurrence in marine habitats is not unusual and probably results from the introduction of freshwater or soil to the marine habitat (Page, 1983). The *Acanthamoeba* sp. isolated in this study was also cultured from farm A. Many *Acanthamoeba* species have been described in marine material, but most will only grow if cultured on freshwater agar but not salt water agar (Page, 1983). This concurs with our findings, the *Acanthamoeba* sp. being isolated and cultured only on freshwater agar. *A. castellanii* has been implicated as the possible causative agent of systemic amoebiasis in the common goldfish (Voelker *et al*, 1977). *A. polyphaga* has been implicated in systemic amoebiasis of blue tilapia, and its ability to produce systemic infection demonstrated in both blue tilapia and carp (Taylor, 1977). An *Acanthamoeba* species has also been implicated in systemic amoebiasis of European catfish (Nash *et al*, 1988). Although pathogenicity attributed to this common genus is widely documented, its isolation in this study has not been considered significant, largely due to its low isolation rate and failure to grow on seawater agar.

A *Vexillifera* species was isolated once in this study. There are no reports of marine species of this genera being pathogenic. However, two freshwater *Vexillifera* species have been isolated from freshwater fish. *V. bacillipedes* has been reported as the causative agent of serious seasonal epizootics of systemic amoebiasis in hatchery-reared rainbow trout from Italy (Sawyer *et al*, 1978). *V. expectata* has also been isolated from the liver of perch but did not seem to be associated with disease or abnormal pathology (Dyková *et al*, 1998a). The isolation of this organism in the present study demonstrates the ability of this genus to colonise fish gills, but was not considered significant.

Of the six *Paramoeba* isolates collected one was classified as *P. eilhardi*, due in part to its large size (45µm-100µm). Due to an inability to maintain this isolate in culture, no attempt was made to undertake electron microscopy, which would have allowed it to be unequivocally identified as *P. eilhardi* due to the distinctive boat shaped “scales” present on the surface of this species (Cann and Page, 1982; Page, 1983). The remaining five *Paramoeba* isolates were consistent with *P. pemaquidensis*³, as described by Page (1983) and Kent *et al* (1988). The *Paramoeba* isolates were most like *P. pemaquidensis* based on the overall size of the isolates, being >25µm, as well as the presence of supernumerary parasomes, a phenomenon not shared by other members of the genus *Paramoeba*, such as *P. aestuarina* (Page, 1983) and *P. invadens* (Jones, 1985). *P. perniciosa*, the agent responsible for ‘grey crab disease’ was also eliminated due to its smaller size, and its failure to be isolated and maintained on any common culture media (Sawyer, 1969; Sprague *et al*, 1969; Newman and Ward, 1973). Page (1983) suggests that the “resolution of species distinctions is likely to require non-morphological investigations”, and suggests the use of isoenzyme electrophoresis. The use of lectin binding sites to differentiate between species of *Paramoeba* has also been investigated, with promising results (Rogerson *et al*, 1992). Use of isoenzyme electrophoresis to differentiate the *Paramoeba* isolates collected in this study from other marine genera was unsuccessful. For the purposes of this thesis the *P. “pemaquidensis”* like isolates collected in these studies was termed *Paramoeba* sp. ‘AGD’⁴.

³ *Paramoeba pemaquidensis* is sometimes referred to as *Neoparamoeba pemaquidensis* as per the reclassification of Page, 1987.

⁴ The identity of a number of the *Paramoeba* sp. ‘AGD’ isolates collected in this study has now been confirmed as *P. pemaquidensis* by 18SrRNA sequencing undertaken by CSIRO in Hobart, Tasmania (see page 173).

Failure to isolate larger numbers of *Paramoeba* isolates may be due to the fact that AGD was relatively mild during the study period, with water temperatures 1-2°C lower than those recorded in years where AGD was more severe (S. Gorman, Pers. Comm.). The culture techniques used to detect amoebae may also have selected against isolating *Paramoeba*. The fact that most isolations of *Paramoeba* were from isopod trails may suggest that competition with bacteria or other amoebae was a factor that reduced the sensitivity of the isolation method. Other amoebae may also have overgrown any *Paramoeba* present, as many of the other marine amoebae, such as *Flabellula* and *Platyamoeba/Vannella* isolates grew faster and adapted better to culture than did *Paramoeba* species. These factors may also explain past problems with the isolation of the causative amoeba in AGD, and also emphasize the need to clone amoebae early in primary isolation. Diatoms were observed growing in conjunction with the *Paramoeba* in four out of six cultures, and it is possible that these organisms may enhance or encourage their growth. Culture of diatoms as a food source was not attempted for maintenance of *Paramoeba* cultures due to perceived interference in the production and testing of specific antiserum, however these organisms may have assisted in the culture of *P. eilhardi*, as suggested by Page (1983). The low isolation rate of *Paramoeba* species has largely been overcome with the modification of an isolation method using ammonium chloride previously described by Jones (1988), as discussed later in this section, however *P. eilhardi* was never isolated again.

3.4.2 Verification of the Pathogen

Immunohistostaining techniques are well established in the diagnosis of a number of human and animal diseases, and are used to show direct association of pathogens to tissue (Polak and Van Noorden, 1984). The value of these techniques is that pathogens are stained *in situ*, using specific antisera and it should thus be possible to identify amoebae associated with gill damage.

The isolates chosen for antiserum production and subsequent immunostaining were selected as representatives of the genera most likely to be involved in AGD. All isolates had been collected from the gills of fish with, or at risk of, developing AGD. *Platyamoeba plurinucleolus* (UQ-1) and *Platyamoeba/Vannella* polymicrobial culture (MP-1) were selected since both cultures had been previously considered to be the pathogen involved in AGD. Furthermore, isolates of this type were the most frequently encountered and isolated genera associated with AGD infected gills. The *Flabellula* isolate (FLB-004) was selected because of its similar morphology and size to amoebae observed in H&E stained histological sections of gill, the frequency with which it was isolated from infected gills at one site, and its postulated pathogenicity (Page, 1983). At least two morphologically distinct forms of *Paramoeba* were collected from gill samples. The isolate chosen for antiserum production was one of the *Paramoeba* sp. 'AGD' isolates (PA-016) as this isolate was similar in size to the organisms seen in H&E stained gill material and the most frequently isolated *Paramoeba* sp. Antiserum to *P. eilhardi* was not generated as it could not be maintained in culture. However, its large size (45-100µm) eliminated it as having an overt role in AGD, as amoebae of this size have not been observed in gill sections. Isolates from other genera were eliminated on the basis of low isolation rate, small size or because their morphology did not resemble the amoebae observed in H&E stained sections.

Immunofluorescence testing of each of the four antisera to each of the four isolates was performed to determine whether the organisms shared common cross reactive antigens which would preclude immunostaining as a means of determining a direct association with the gill. No cross reactions were observed suggesting that the antiserum was specific at the concentrations tested.

Antiserum to *Paramoeba* PA-016 reacted strongly with all five *Paramoeba* sp. 'AGD' isolates tested, suggesting that they were a homogenous group sharing common antigens. No reaction was observed when this antiserum was tested against 42 other amoebae isolated during the study, suggesting that the antiserum was highly specific to *Paramoeba*. Recently antiserum to another *Paramoeba* isolated from AGD

infected salmon during these studies (PA-027) was tested against members of the genus *Paramoeba*, from type culture collections, to further determine the specificity of the antiserum (Douglas-Helders *et al*, 2001). The antiserum reacted with *P. pemaquidensis*⁵, as well as the near related species *N. aestaurina* (formerly *P. aestaurina*) and *Pseudoparamoeba pagei*, suggesting the presence of a common antigen amongst these related species of *Paramoeba*. The cross reactivity of the *Paramoeba* antiserum with *N. aestaurina* and *Pseudoparamoeba pagei* is interesting but does not diminish the results of present in this present study, as both species are considerably smaller than *Paramoeba* sp. ‘AGD’ (J. Carson, Pers. Comm.), and neither were identified in this study. The antiserum did not react with *P. eilhardi*; this finding was not surprising since the taxonomic position of *P. eilhardi* had long been in question due to its size and its distinct cell surface, leading Page (1987) to separate it from other *Paramoeba* into the amended family, Paramoebidae. Under the revised classification *P. pemaquidensis* and *P. aestaurina* were placed into the family Vexilliferidae, and the genus *Neoparamoeba*.⁶

Immunofluorescence was chosen to screen the gill samples as it is a simple and sensitive technique enabling rapid detection of whole organisms in fixed or frozen tissue. Reactive amoebae stain an intense fluorescent yellow/green against a non-fluorescent background. Tissue detail cannot be clearly observed and the preparation is not permanent. In this study, fixed and frozen gill samples known to be positive for amoebae by H&E staining were screened using antisera made to the four distinct amoeba isolates in order to determine what role, if any, each of the isolates played in AGD. The technique employed in this study was validated by testing brain tissue of rainbow trout infected with *Streptococcus* sp. with a specific antiserum, this method also acting as a positive control.

⁵ Also known as *Neoparamoeba pemaquidensis*

⁶ The revised classification was not used in this study as the key did not provide detailed morphological characteristics (as in Page 1983), and as the position of *P. invadens* and *P. perniciosa* in the reclassification was not known.

Large numbers of fluorescent amoebae were observed when gill samples, known to be positive for amoebae, were immunostained with *Paramoeba* sp. 'AGD' antiserum. Failure to detect *Paramoeba* in one of the 37 samples was probably due to the small number of amoebae present in this sample, or the histology section used having been cut past the affected area. Very few of the same sections showed fluorescent amoebae when immunostained by the other three antisera; the number of fluorescent amoebae in the positive sections were small, ranging between 1-3 organisms. Fluorescence was also weaker than with *Paramoeba* antisera.

The results of immunofluorescence screening verify the pathogen associated with AGD as a *Paramoeba* species, and excluded any overt involvement by other amoebae in AGD. As *P. eilhardi* was unable to be maintained in culture its involvement, if any, in AGD could not be established. However, since the predominance of amoebae in gill sections from fish with AGD fluoresced with *Paramoeba* sp 'AGD' antiserum, and this anti-serum does not cross react with *P. eilhardi* (Douglas-Helders *et al*, 2001), it is unlikely that *P. eilhardi* plays any overt role in the development of AGD. The results suggest that other amoeba species are able to colonise the gills of AGD affected fish; but are not present in the numbers sufficient to account for the numbers present on the surface of affected gill tissue.

Large numbers of fluorescent amoebae were observed in frozen sections immunostained with *Paramoeba* sp. 'AGD' antisera, many not attached to the damaged gill. Frozen gill samples were originally collected, as it was not known if chemically fixed gill material was suitable for immunostaining as it was thought that formalin and Davidson's fixatives might alter the antigenicity of amoebae in gill tissue. Significant loss of antigenicity was not observed in the tissues tested. Treatment of chemically fixed gill tissue with protease to expose antigenic sites did not appear to enhance staining and hence antigenicity, although this may be chemical dependant and have to be determined empirically. Amoebae in chemically fixed gill material are known to detach from the gill during fixation, the rate being dependant on the fixative used. The occurrence of large numbers of attached and unattached *Paramoeba* in frozen material, compared to chemically fixed material collected at the

same time, suggests that more amoebae are retained during freezing. Hence the number of *Paramoeba* present on the gills of fish with AGD is likely to be grossly underestimated using standard H&E stained gill tissues, and in fixed tissues stained by immunofluorescence. However, the collection of frozen tissue for routine analysis is not justified or practical due to logistical problems associated with snap freezing gill tissue in the field, frozen sectioning being labour intensive, and the quality of the gill tissue after sectioning being inferior to chemically fixed tissue.

Comparison of the immunofluorescence technique using *Paramoeba* sp. 'AGD' antiserum to standard H&E stained gill sections was undertaken to determine the relative sensitivity of these two techniques in detecting *Paramoeba*. The immunofluorescence technique was more sensitive in detecting low numbers of *Paramoeba* in gill tissue (19/22) than histological examination of H&E stained material (7/22), indicating that this technique may be useful in detecting cases of mild or early AGD.

The success of the immunofluorescence testing indicated that it might be possible to adapt this technique into a diagnostic technique to determine the extent of AGD in populations of sea-caged Atlantic salmon, the development of which will be discussed in Chapter 6.

The presence of different types of amoebae, including *Paramoeba* species, in isopod sections supports earlier work where amoebae were isolated from isopod trails on agar plates. The presence of moderate numbers of *Paramoeba* associated with a frozen section of isopod suggests that *Paramoeba* may be commensal organisms of isopods. Alternatively, the isopods may have acquired the amoebae whilst on the surface of the gills. The role of isopods in AGD remains unclear; they may act as a reservoir for the pathogen or act as a vector/transport host transferring *Paramoeba* from infected fish to uninfected fish. If the latter is the only role for isopods in the disease, the extent of its role in transmission of AGD and its contribution to spread of the disease remains to be determined. However, subsequent field work has shown that infestation with isopods is variable, with little infestation being observed in some seasons (D. Cameron, Pers. Comm.), AGD however occurs every summer season

suggesting that while these isopods may exacerbate disease they are not a primary reservoir of *Paramoeba* infection. Sea lice (*Leptophtheirus salmonis*) are a significant parasite of salmonids in other sea-farming countries, and a related species to isopods. They have been implicated in the spread of bacterial diseases of salmonids (Cusack and Done, 1986; Nylund *et al*, 1991 and Lars and Øivind, 1993). These studies have demonstrated the presence of bacteria on and in the digestive tracts of sea lice found on salmonids, including the causative agent of furunculosis, *Aeromonas salmonicida*, a disease that causes severe losses in cultured fish in many parts of the world. Sea lice were also shown to transmit the virus infectious salmon anaemia (ISA) to salmon smolt when challenged with lice from ISA infected fish (Nylund *et al*, 1993). Although the ISA virus can be transmitted without the presence of sea lice, the mortality in fish infected with lice was higher than in fish not infected (Nylund *et al*, 1994).

The presence of organisms reactive to *Paramoeba* sp. 'AGD' antiserum in gill samples from New Zealand and France with similar histopathology to AGD, has suggested that the same or a related *Paramoeba* species is responsible for AGD in salmonids farmed in other countries. Identification of this organism in gill tissue from France constitutes the first report of this infection in the European salmonid industry. Gill sections from an outbreak of AGD in Atlantic salmon in Ireland have also been tested positive using the *Paramoeba* sp. 'AGD' antiserum (Rodger and McArdle, 1996), but were negative when tested with the other three antisera prepared in this study (Palmer *et al*, 1997). These results suggested that the *Paramoeba* species responsible for AGD in salmonids of other sea-farming countries is similar, if not the same, as the species found in Tasmanian salmonids. This has now been confirmed by 18S rRNA sequencing undertaken by the CSIRO in Hobart, Tasmania. Five *Paramoeba* isolates collected during the present study were compared to four reference strains of *Paramoeba pemaquidensis* (including the *Paramoeba* isolated in the USA from AGD infected coho salmon by Kent *et al* {1988}), and an isolate from Ireland. The sequence similarity of all isolates was found to be >98% and confirms the identity of the Tasmanian isolates as *P. pemaquidensis* (F. Wong, N. Elliot, J. Carson, Pers. Comm.).

The presence of AGD and *Paramoeba* in harvest-sized salmon constitutes the first unequivocal evidence of AGD in winter, contradicting anecdotal reports that the disease occurs only in summer. These fish had not been bathed during the previous summer, and may have carried the infection over into winter. No significant losses were reported, which may be because they were harvested immediately the disease was noted (J. Smith, Pers. Comm.). While AGD may be more florid during the summer, evidence of infection in mature fish during the winter raises some doubt regarding the development of protective immunity in older fish. Alternatively, the infection may have remained subclinical until conditions of stress (e.g. high water temperatures) allow the pathogen to proliferate, leading to AGD.

After initial immunofluorescence screening, selected sections were immunostained with *Paramoeba* sp. 'AGD' antiserum using the immunoperoxidase method. The immunoperoxidase staining technique was employed to allow *Paramoeba* to be observed in direct association with stained gill tissue. This technique is more time consuming but allowed tissue detail to be observed as well as the target organism. The stained sections are permanent allowing detailed analysis of the organism and the gill tissue, as well as direct comparison with H&E stained material. An added advantage of this method was that the sections could be viewed by light microscopy. Other antisera were not screened as the results of the immunofluorescence screening excluded overt involvement of these genera.

In immunoperoxidase stained sections, *Paramoeba* were often observed in large numbers in gill sections in association with gill damage in fish with AGD. Amoebae were often observed lining the lamellae of the affected gill, encrypted in the damaged tissue and penetrating the gill epithelium. The association of large numbers of *Paramoeba* with damaged tissue and their proximity to that damage adds further weight to the pathogenic role of this organism.

Alkaline phosphatase produced a better contrast between the gill and amoebae than did peroxidase staining, although the choice of enzyme stain is purely esoteric. It is however a little more time consuming and more expensive than the immunoperoxidase method.

Leiro *et al* (1998) criticised the use of immunostaining in the present study, suggesting that “it would be difficult to rule out the possibility of cross-reactivity with other free-living amoebae, and because the technique would not distinguish between association and primary cause”. Although the sera used to probe these histology sections were polyclonal, significant investigation was undertaken to ensure the polyclonal antisera were specific, especially in relation to the *Paramoeba* antiserum, which did not cross react to 42 other marine amoeba isolates. The likelihood that the *Paramoeba* antiserum is reacting to another marine amoeba is remote. The authors suggested the use of ELISA to investigate whether sera from diseased fish bind to the cultured amoebae, this was attempted and proved, and will be discussed in Chapter 7. The development of a *Paramoeba* sp. ‘AGD’ specific monoclonal would provide the best means of supporting the conclusions made here, but was not attempted in the present study, due in part to a lack of adequate laboratory facilities and in most part to the time needed to produce a monoclonal versus the perceived benefit to the overall study objective. However, the development of specific *Paramoeba* sp. ‘AGD’ monoclonals warrants further investigation, as their development may make a significant contribution to vaccine development, and may increase the sensitivity of any diagnostic assays developed (see Adams *et al*, 1995). The present study however agrees with Leiro *et al* (1998) that the only conclusive way of demonstrating that the cultured organism is responsible for the observed disease would be experimental infection. Experimental infection using *Paramoeba* sp. ‘AGD’ was investigated in Chapter 4.

There is no compelling evidence to suggest that any other pathogen is involved in AGD in salmon or turbot. Amoebic gill disease is not systemic, and there have been no reports of histopathological changes in the internal organs (Kent, 1988; Munday *et al*, 1990; Dyková *et al*, 1995; Rodger and McArdle, 1996;). Significant pathological changes are restricted to the gills. Munday *et al* (1993) reported the presence of bacterial colonies in some gill lesions in AGD affected fish, but these did not resemble the palisades of organisms seen in bacterial gill disease. Dyková *et al* (1991) found only one single bacterial embolus in hundreds of examined gill histological

sections, suggesting an insignificant role of bacteria in the development of AGD in turbot. Small numbers of bacteria have been observed in AGD affected gill sections examined by transmission electron microscopy (TEM), most of which were observed in the cytoplasm of the gill-attached amoebae (Roubal *et al*, 1989; Dyková *et al*, 1998). These TEM studies add further weight to the lack of overt involvement of bacteria AGD, as larger numbers of bacteria would have been expected in the surrounding gill tissue. In Tasmania, extensive passive surveillance has failed to find any consistent bacterial findings in the gills of AGD affected salmon, again suggesting that bacteria do not play an overt role in AGD (J. Carson, Pers. Comm.). However, there is some evidence to suggest a covert role for bacteria in AGD. Roubal *et al* (1989) were the first researchers to question the role of gill-surface dwelling bacteria in AGD, although these researchers failed to find any microscopic evidence of large bacterial loads prior to the presence of AGD. Cameron (1993) studied the bacterial load on the gills of smolt following transfer into the sea, finding a progressive increase in the number of bacteria with time post-transfer to the sea (presumptive identification of *Flexibacter/Cytophaga* and *Vibrio* spp.). Indeed, the author reported that this increase was ‘indicative of rapid colonization and proliferation of marine bacteria immediately following transfer’. After this initial period a slower but steady increase in bacterial numbers was observed. Cameron (1993) suggested that the bacterial load might result in the development of conditions favourable for the proliferation of *Paramoeba*. Roubal *et al* (1989) suggested that if bacteria were found to favour the development of AGD, management strategies could concentrate on reducing the levels of bacteria by prophylactic antibiotics or bath treatments. To date there have been no further studies of the role of bacteria or bacterial load in the development of AGD, in either turbot or Atlantic salmon.

Other organisms have been found in the gills of AGD affected fish. Kent *et al* (1988) reported the presence of the microsporidian *Loma salmonae* and the gill monogenean *Laminisus strelkowi* in the gills of some AGD affected fish; however these findings were not consistent as amoebae were the only pathogen detected in other salmon with AGD from the same epizootics. Rodger and McArdle (1996) also reported the presence of *Trichodina* species in Atlantic salmon with AGD, but this was not

consistent. In this study the presence of diatoms and other unidentified motile protozoa was observed during primary isolation of amoebae from the gills of salmon with and at risk of AGD (unpublished results), but again these findings were not consistent. It is most likely that these organisms are incidental, either taking advantage of the damaged gill tissue, or present in the sea-water surrounding the gills at the time of sampling. There are no reports on the investigation of viral pathogens in the gills of AGD affected fish. In Tasmania, anecdotal evidence suggests that the absence of viruses in the gills of AGD affected fish, however no comprehensive studies have been performed to eliminate this possibility. Again, extensive passive surveillance in Tasmania has failed to find any histological evidence for a viral agent contributing to AGD, and limited TEM studies on the gills of fish at risk of AGD have not found any viral pathogens present (J. Handler, Pers. Comm.). Further investigation is required if a co-infection with a viral agent is to be ruled out as a contributing factor in AGD. The presence of other pathogens, apart from amoebae, was not investigated during this study. However in the hundreds of gill histological sections examined during this study there was no evidence of any overt bacterial or protozoal infections (aside from the presence of amoebae), and no viral inclusions were recorded.

3.4.3 Gill health

The study of gill health over this period was performed primarily to relate amoeba isolations to the level of AGD observed, and to provide fixed gill sections to compare H&E staining with immunostaining. However, examination of gill health over this period has provided additional information regarding the progression of disease.

Gross gill health was examined as a way of assessing the level of AGD observed at the time of sampling. It also enabled the isolation rate and types of amoeba to be compared over the disease period. As reported, the levels of mucus and number of mucous patches increased during the waves of infection, indicating a progression of AGD. Histological examination of gill sections showed a progression of gill damage or irritation, leading to typical AGD lesions and also demonstrated the presence of amoebae. Damage or irritation in the gills was observed very early after the fish were

transferred to the sea, before amoebae could be detected microscopically. This damage was characterised by the presence of small lymphoid nodules and early fusion of the secondary lamellae. Progression of the damage was characterised by an increase in the number and size of the lymphoid nodules, and larger areas of partial and total fusion of the secondary lamellae (pre-AGD lesions), with few amoebae. Gills from fish with AGD showed further progression of gill damage characterised by larger areas of fusion, pseudostratification of the epithelial cells, and the presence of small numbers of amoebae. The progression of gill damage leading to AGD is in agreement with observations from other researchers (Nowak and Munday, 1994).

The absence of amoebae in gill sections with early signs of gill damage suggests that the damage may be present before the gills are colonised with *Paramoeba*⁷. It should be noted, however, that the infected fish used in the study were experimentally infected in tanks, and it is not known if the progression of AGD in tanks is indicative of normal field exposure. In the present study immunofluorescence staining of gill tissue with the anti-*Paramoeba* antibody was more sensitive in detecting low numbers of *Paramoeba* in gill sections than routine histological examination. This suggests that routine histological examination of gill tissue may underestimate or miss the presence of *Paramoeba* in the tissue. Alternatively the absence of amoebae in these early sections may be due to the fixation method used, as amoebae are known to detach from the gills during fixation. This was demonstrated in the present study when frozen tissues were examined, showing much larger numbers of *Paramoeba* present, many not attached to the damaged gill. It would be useful to undertake a sequential study of Atlantic salmon gill health using frozen gill tissue, since more amoebae may be retained during the freezing process. Differences in gill tissue integrity and amoebae retention was observed between fixatives used in this study, a phenomenon that was examined in Chapter 6.

⁷ Recently Zilberg *et al* (2000) found *Paramoeba* sp. attached to normal gill tissue, as identified by immunohistostaining (using a modification of the technique described in this study), suggesting that the gills do not have to be damaged to allow infection to occur.

Overall, the number of *Paramoeba* isolated and observed in gill sections stained with H&E and those immunostained were small. It has been suggested that the damage caused by *Paramoeba* in the fish gills may be due to a heightened allergic response (B. Munday, Pers. Comm.) or due to a parasite excretory factor (Roubal *et al*, 1989), requiring only a small number of amoebae. If the severity of AGD is a result of hypersensitivity, the failure by past researchers to isolate the putative pathogen *Paramoeba* sp. 'AGD' and the low isolation rate in this study may be due to the low numbers of *Paramoeba* present in the gills initially, and the interference of other opportunistic amoebae after damage has occurred.

Freshwater bathing is an effective means of controlling AGD. Examination of post-bathed fish showed a reduction in the amount of mucus and number of mucous patches. Total eradication of the patches was achieved in fish bathed during 'wave' 1 of infection, but was not achieved in fish bathed in 'wave' 2, where only a reduction in the number of patches was seen. These results suggest that the disease had progressed further in 'wave' 2, and that the therapeutic benefit of freshwater bathing was reduced. The failure of freshwater bathing to totally eradicate mucous patches in later waves of infection may explain why progressive 'waves' of infection were shorter. However, histological examination of H&E stained gill material confirmed that freshwater bathing caused a reduction in the amount of mucus and the removal of amoebae. These results seem to contradict reports in which the second freshwater bath was observed to have a longer lasting effect on the prevalence of AGD lesions with *Paramoeba*, suggesting the development of acquired resistance to AGD (Findlay and Munday, 1998; Clark and Nowak, 1999). However these inconsistencies may be due to the mild conditions during wave 1 in this study, and the small numbers of fish sampled.

Gross examination of the gills undertaken during sampling period demonstrated that the level of isopods in the mouth and on the gills of Atlantic salmon increased during waves of infection, coinciding with the development of moderate to severe AGD, adding to the speculation that they may play a role in spread of AGD. Examination of isopod sections demonstrated a variety of commensals or parasites associated with the

isopods, also direct evidence of host gill tissue in the mandibles of the isopods. Amoebae were not seen in chemically fixed sections, probably as a result of the fixation methods used. However, a moderate number of amoebae were observed on frozen isopod sections when immunostained with *Paramoeba* specific antisera. The role of isopods in exacerbating AGD is currently unknown and warrants further investigation.

The number and diversity of amoebae isolated from fish gills increased proportional to the degree of damage to the gills, suggesting that many other marine amoebae, besides *Paramoeba* will temporarily take advantage of the damaged gills without overt involvement in the initial disease. The results also demonstrate the difficulties experienced by past researchers when attempting to isolate *Paramoeba* from the gills of fish with AGD .

The “mushy” gill pathology observed during this study (Figure 3.7) was attributed to a new syndrome, which was described after the sampling period, called clubbing and necrosis gill syndrome (CNG syndrome) (Clark *et al*, 1997). The gross gill pathology was similar to AGD, in that the gills showed the presence of excess mucus, but no discrete mucous patches. Histopathology of the gills was also similar, however there were no amoebae in the gills, and the fish did not respond to freshwater bathing. Isolations of amoebae from the gills of 15 affected fish produced a diverse range of marine amoebae, but no *Paramoeba*, the number and diversity being proportional to the damage observed. This syndrome was later found to be an entirely new disease, which does not involve an amoeba species. Some involvement of toxic marine algae was suspected, but to date no pathogen has been found to be associated with CNG syndrome (Clark *et al*, 1997). This work is not reported in this thesis, its inclusion here demonstrates that marine amoebae of genera other than *Paramoeba* colonise damaged gill tissue, regardless of the primary cause of the damage. These marine amoebae do not appear to proliferate on the gills, adding further evidence that do not play any overt role in the development of AGD. Further investigation is required to determine why *Paramoeba* sp. ‘AGD’ proliferates on the gills of AGD affected fish, over that of other marine amoebae.

3.4.4 Additional Investigations

Methods for the long-term storage of amoeba isolates were investigated to provide a reference library of marine amoeba isolates present on salmonid gills, particularly *Paramoeba* sp. 'AGD', and ultimately to protect against the selection of laboratory adapted strains. Long-term preservation at -70°C using the cryopreservation medium was successful in allowing long term storage of most of the marine amoeba isolates collected. This method was chosen primarily because a similar method has been successfully used by the State Water Laboratories in South Australia for preservation of a large number of freshwater amoebae genera (B. Robinson, Pers. Comm.). For this reason it was thought that this method would be similarly successful in a large range of marine genera. Although freezing in sterile sea-water alone and with DMSO was successful for the one isolate investigated it is not believed to be the optimal freezing method for all marine amoebae genera, although this has not been investigated. These methods may be useful in freezing those few isolates that were unable to be frozen by the cryopreservation media method.

It is not known why selected isolates were unable to be frozen by the cryopreservation medium method, despite some of these isolates being of the same genus as some that were successfully frozen. Perhaps the health of these cultures was not optimal, the plates may have been too dry and the amoebae more susceptible to osmotic shock, or that they had exhausted the bacterial substrate and were close to death.

Paramoeba species were unable to be successfully frozen, despite using apparently healthy cultures and numerous methods of freezing and retrieval. Although the *Paramoeba* cells appeared intact on retrieval, none were viable. For this reason *Paramoeba* cultures could not be protected from laboratory adaptation and loss of virulence, and therefore isolation of *Paramoeba* was continued throughout the rest of the study period to provide younger cultures for research with these cultures being maintained by regular subculture. The inability to freeze *Paramoeba* species is supported by the Culture Centre of Algae and Protozoa, where maintaining these species is also by regular subculture (J. Carson, Pers. Comm.). However the American

Type Culture Collection (ATCC) freezes *Paramoeba* species, suggesting they have a viable method, however the details of this method have not been made available (J. Carson, Pers. Comm.). During this study an isolate of *Paramoeba pemaquidensis* was obtained from the ATCC by Dr Bret Robinson (State Water Laboratory, South Australia) on behalf of this study; all attempts to culture this isolate failed. No further attempts to obtain type cultures were made during this study.

The number of *Paramoeba* species isolated in this study was low compared to the isolation of other genera of amoebae. As a *Paramoeba* species has been identified as the amoeba associated with AGD, it became important to develop an isolation method that would increase the isolation of this genus. The success of the sea-water wash method in isolation of marine amoebae lead to the investigation of a method previously developed to remove *Paramoeba* from the gills of Atlantic salmon for enumeration (Jones 1988). Alexander (1991) investigated this method to quantitate the numbers of amoebae on the gills of fish undergoing various medicated treatments and found it to be too variable for this use. However investigation of this method to isolate amoeba and paramoebae from the gills of fish have shown its efficiency as an isolation method. The efficiency of this method is probably due to a more effective removal of the paramoebae, and other marine amoebae, from the gill tissue. The use of malt yeast agar plates and *S. maltophilia* as a growth substrate may have also contributed to the higher isolation rate. Subsequent isolations using this method have shown its efficiency in isolating many amoebae from infected fish, and in all cases of amoebic gill disease *Paramoeba* sp. 'AGD' have been isolated. Despite these improvements in isolation *P. eilhardi* has never been isolated subsequently.

The need to confirm the identity of isolated marine amoebae is of paramount importance given the diversity of isolates collected from fish with AGD. Past studies of AGD have been hindered by the use of isolates not unequivocally identified as *Paramoeba* sp.. *Paramoeba* sp. 'AGD' is slower growing than many other isolates, and often more fastidious, therefore identification and cloning of isolates is important. Preliminary identification of isolates at the initial culture step, eliminates many amoebae as not belonging to the genus *Paramoeba*, based on the growing

characteristics observed. Those isolates with growth characteristics consistent with *Paramoeba* can then be examined for the presence of a parasome. A number of methods were investigated to confirm the presence of a parasome, allowing laboratories without long focal length microscopes or fluorescent microscopes to confirm the identity of suspected *Paramoeba* isolates. The method of choice for our laboratory was the DAPI method that is a rapid technique, and unequivocally demonstrates the presence of parasomes. This method was used periodically to check the integrity of cloned *Paramoeba* isolates throughout the course of these studies. Phase contrast microscopy of amoebae on agar slides and the Gomori's trichrome stain were also successful methods, although slightly more time consuming. Phase contrast microscopy of amoebae on agar slides being most useful in the field when microscopes with phase contrast objectives are available. Overall the identification of *Paramoeba* isolates is now a rapid process using these methods, greatly reducing the time spent processing primary isolation cultures, and allowing greater confidence in the isolates being used for drug and pathogenicity trials.

3.5 CONCLUSIONS

Immunostaining of gill sections, known to be positive for amoebae, with antiserum made to amoeba isolates collected during an extensive sampling programme, demonstrated that a *Paramoeba* species is the amoeba associated with gill damage in fish with AGD. Very few sections showed specifically stained amoebae when immunostained by antisera made to a number of other amoeba isolates isolated from the gills of fish with or at risk of AGD. These results verified the pathogen associated with AGD as a *Paramoeba* species, and excluded the overt involvement by other amoebae in AGD. The *Paramoeba* isolates were morphologically consistent with *P. pemaquidensis* (Page, 1983; Kent *et al*, 1988) and was termed *Paramoeba* sp. 'AGD'.

Fixed gill samples from New Zealand and France were immunostained with anti-*Paramoeba* sp. 'AGD' antiserum and tested positive, suggesting that the same or related *Paramoeba* species is responsible for AGD in salmonids farmed in other countries. Identification of this organism in gill tissue from France constitutes the first report of this disease in the European salmonid industry.

Isolation of *Paramoeba* from isopods, and the detection of *Paramoeba* in isopod sections implicates them as either a reservoir of infection, or as a vector transferring *Paramoeba* from fish to fish. Although not a primary reservoir of infection, the role of isopods in transferring infection and exacerbating AGD requires elucidation.

Histological examination of gill sections showed a progression of gill damage consistent with other researchers (Nowak and Munday, 1994). However, the presence of AGD in the gills of harvest-sized fish in winter constitutes the first unequivocal evidence of AGD in winter, contradicting anecdotal evidence that AGD only occurs in the warmer months, and raising some doubts regarding the development of protective immunity in older fish.

Initially the number of *Paramoeba* strains isolated during the sampling period was low compared to the isolation of other amoeba genera. The ammonium chloride wash method adapted from the method described by Jones (1988), combined with refined culture conditions led to a vast improvement in the isolation rate of *Paramoeba* strains, resulting in the isolation of *Paramoeba* in all cases of AGD. However, the number of other amoebae genera also increased, and this led to the development of identification criteria that would distinguish *Paramoeba* sp. 'AGD' by its' growth characteristics. Improvements made in the identification and confirmation of *Paramoeba* isolates significantly reduced the time taken to process primary isolation cultures. Cryopreservation was successful with all marine and freshwater amoebae isolated and maintained during this study, except *Paramoeba*.

Verification of the amoeba responsible for AGD, combined with improvements made in the isolation and confirmation of *Paramoeba* isolates now allows greater confidence in the isolates being used in pathogenicity and chemical trials. In addition the antiserum produced to *Paramoeba* sp. 'AGD' had the potential to be used to develop a simple and specific diagnostic assay to detect amoebae in clinical material.

Chapter 4. Pathogenicity of *Paramoeba* sp. ‘AGD’

4.1 INTRODUCTION

There is good evidence to suggest that a *Paramoeba* species is the aetiological agent of amoebic gill disease (AGD) in salmonids, based on gill histopathology where large numbers of *Paramoeba* are observed associated with gill damage, some with pseudopodia penetrating the surface epithelial cells of the gills; and immunostaining of gill tissue with *Paramoeba* antiserum which show large numbers of specifically stained *Paramoeba* associated with damaged gill tissue. However, there is a need to verify the descriptive reports of pathogenicity ascribed to *Paramoeba* species.

Attempts to infect naïve coho salmon in the US and naïve rainbow trout in Tasmania with *Paramoeba* cultured from the gills of infected fish were unsuccessful (Jones, 1988; Kent *et al*, 1988; L. Searle, Pers. Comm.). There is some evidence that naïve rainbow trout exposed to naturally infected fish subsequently develop gross signs of AGD (L. Searle, Pers. Comm.), but this method has not been defined.

Isolation of authentic strains of *Paramoeba* and the development of methods to cultivate and maintain *Paramoeba* has allowed pathogenicity trials to be undertaken, in an attempt to fulfil Koch’s postulates. The development of a model of infection would provide a means of carrying out, irrespective of season, controlled studies of pathogenicity and tank trials of treatments for AGD. In this study, two models were investigated: exposure of naïve fish to cultured *Paramoeba* to precipitate AGD, and fulfil Koch’s postulates; and to verify and optimise the infection of naïve fish using AGD infected fish.

In addition, cultured *Paramoeba* were examined by electron microscopy for the presence or absence of fine hair-like filaments on the surface of the cells. To date these surface hairs have been reported on gill-attached amoebae, but not in cultured *Paramoeba* (Kent *et al*, 1988; Roubal *et al*, 1989; Munday *et al*, 1990). The presence or absence of these hair-like filaments hairs was the only clue in the literature to a possible virulence factor.

4.2 METHODS

4.2.1 Tank systems

During the course of these studies two tank systems were devised to challenge naïve fish with cultured *Paramoeba* sp. ‘AGD’, the latter being improvements resulting from limitations of the original system. Tank system 1 was set up as a flow-through system. In tank system 2, one tank was set-up as a recirculation system, the other two were set up with flow-through facilities as in tank system 1. Five culture challenge experiments were conducted, the first experiment using tank system 1, the later challenges with the recirculation tank of system 2. Two cohabitation challenges were conducted using the flow-through system for both challenges. The tanks were located at Saltas Marine Operations, in Dover, Tasmania.

Tank system 1 - flow-through system

This system consisted of three 2m² fibreglass tanks (Figure 4.1), filled to a depth of approximately 0.5m to give a nominal operating volume of 2000L. Water entered the tanks through valves situated above the water line at the edge of each tank and discharged via a screened sump (stainless steel; 5mm holes) in the bottom centre of each tank. Water level was controlled by an external (effluent) standpipe (Figure 4.2).

Sea-water was drawn from a depth of three metres, 20 metres off-shore, through 40mm polyethylene piping by a small pressure pump. Sea-water was either pumped directly to the experimental tanks (ambient water) or diverted into a header tank, where it was heated and gravity-fed to each tank as required (Figure 4.3). Flow rates through the system were kept at 10-15L/min to keep the water in each tank at the desired temperature, and the concentrations of potential toxic metabolites such as ammonia below toxic levels (S. Oddsson, Pers. Comm.). Additional oxygen was supplied to each tank through 15cm diffusers, nominal pore size 0.4µm (Point Four System Inc. Canada) to maintain oxygen concentration at >7 ppm, as measured in the effluent water.



Figure 4.1: Tank system 1, showing three fibreglass tanks.

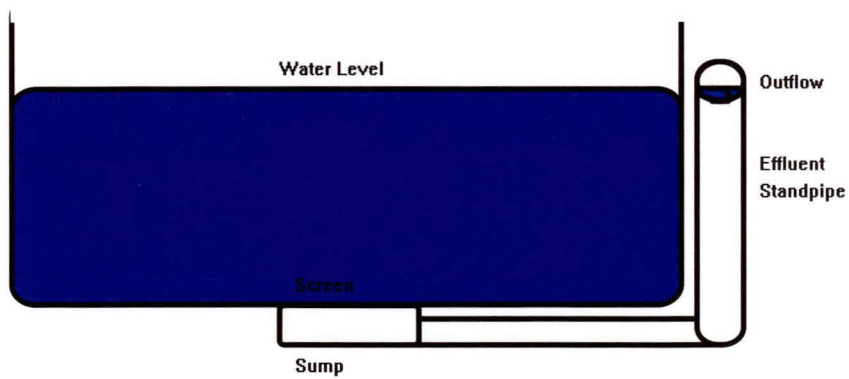


Figure 4.2: Schematic side-view of tank showing effluent water system

Tank system 2 - recirculation system

Two of the tanks from the flow-through system were replaced with 1m² fibreglass tanks with the same design as the 2 metre square tanks. The operating volume of these tanks was 700L. The remaining 2m² tank was retained to house fish for experimentation and the control fish. The large tank and one small tank were set-up as flow-through tanks, as described for tank system 1. Sea-water supplied to these tanks was at ambient temperature and was not heated. The remaining small tank was set-up with recirculation.

For the recirculation system one tank was connected to a 500L biofilter tank containing 300 high surface area balls (Bioballs, Academy Plastic), the biofilter was seeded with a commercially prepared mix of nitrifying bacteria (AdMac Agencies, WA.). This biofilter was employed to remove and neutralise toxic metabolites that tend to build up in the recirculating water. Sea-water was pumped as already described, except filtered through 10, 5 and 1µm cartridge filters. Filtered sea-water was pumped into the recirculation tank and biofilter. From the connected biofilter, water was gravity fed to a 100L sump then pumped to a 600L header tank, where the water was heated to 18°C and pumped directly back into the recirculation tank. The approximate working volume of this system was 2,200 litres. Tanks were flushed daily to remove detritus, by removing 25 litres of sea-water, the volume replenished with filtered sea-water. Unless stated otherwise no additional oxygenation was supplied. As only one recirculation system was available no control fish could be run with the culture challenges. Figure 4.4 depicts the set-up of tank system 2.

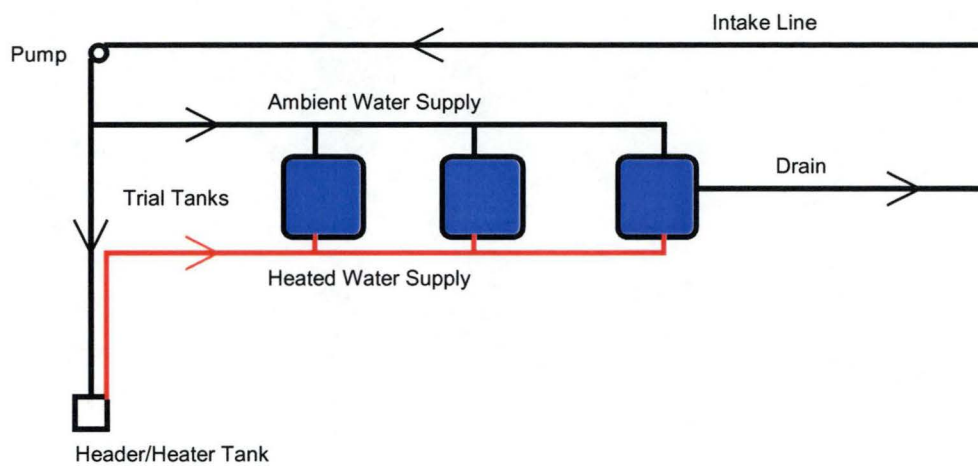


Figure 4.3 Schematic plan of tank system 1.

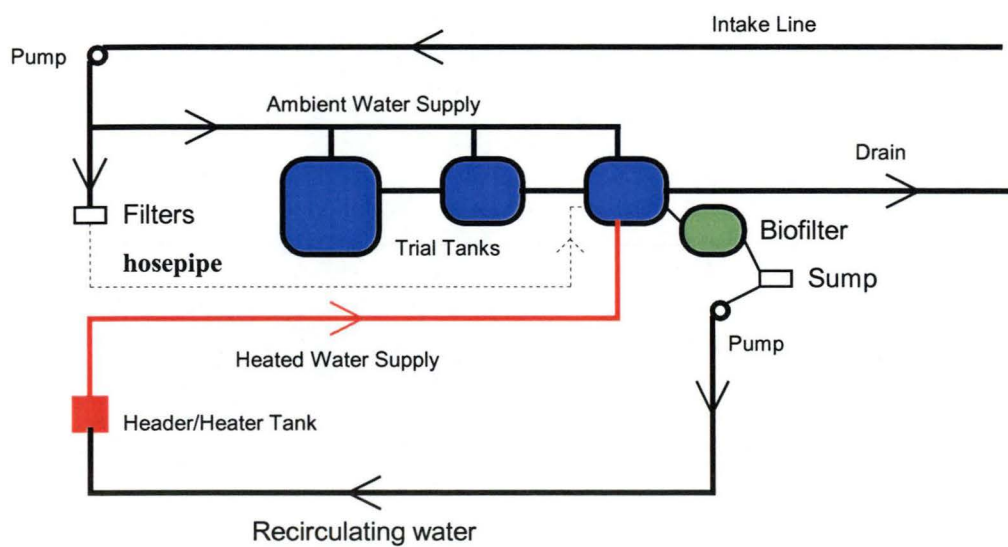


Figure 4.4 Schematic plan of modified tank system 2.

4.2.2 The fish

The first culture and cohabitation challenges employed rainbow trout (*Oncorhynchus mykiss*), maintained in freshwater under commercial hatchery conditions until challenge. Prior to acclimation to sea-water, the trout were fed on a diet supplemented with 5% (w/v) sodium chloride for at least two weeks. Fish were acclimated to full sea-water over a 10 day period. They were then placed in 10 ppt sea-water and the salinity increased by 5 ppt every second day. At the time of transfer, the trout weighed between 120-150 grams each. Rainbow trout smolt were kindly donated by Russell Falls Aquaculture Pty Ltd.

The remaining culture and cohabitation challenges were conducted using Atlantic salmon smolt. Atlantic salmon were grown and maintained in freshwater, under commercial hatchery conditions, prior to transfer to sea-water. Salmon smolt were not acclimated to sea-water, they were however fed on a diet containing 5% (w/v) sodium chloride for at least two weeks. The fish for these challenges weighed between 350-700 grams. Atlantic salmon were supplied by SALTAS's commercial hatchery, located at Wayatinah.

AGD infected salmon were obtained from the SALTAS marine research farm located at Dover. The size of fish was between 700 grams and 1.5 kilos. Fish were collected and scored for the level of AGD by assessing the number of mucous patches, as described in Method 2.6.1, prior to addition to the tanks.

Husbandry

Daily maintenance involved feeding with standard salmon feed; monitoring and adjusting as necessary, water flows, temperature and oxygen levels; flushing tank sumps to remove detritus; and the removal of any dead fish. Flow rates were adjusted to accommodate the number of fish present in each tank and to maintain a steady temperature. Temperature, oxygen levels (mg/litre) and feeding behaviour were recorded daily. Dead fish were removed from each tank daily, the number recorded and disease assessed as described below.

4.2.3 Disease assessment

The development of AGD in the challenged fish was assessed using a number of methods. All fish were examined for signs of gross gill pathology, indicated by the presence of mucous patches as described in method 2.6.1. Gill samples for histological examination were taken periodically. Fish were euthanased by overdose with benzocaine and gill samples collected in chemical fixatives for histological examination (Method 2.6.2); samples were most often collected in sea-water Davidson's (Appendix 6.3), isotonic formalin (Appendix 6.2) and Bouin's fixatives (Appendix 6.5). Gill smears were also taken for immunofluorescence testing by the *Paramoeba* IFAT (Method 2.6.3), the development of which is discussed in Chapter 6. Gill smears were not taken for culture challenge 1 and cohabitation challenge 1 as this technique had not yet been developed. Microscopic examination of the gill sections were conducted by Dr Judith Handlinger of the Fish Health Unit, DPIWE, Launceston, Tasmania.

4.2.4 Culture challenges

Culture and preparation of *Paramoeba*

Growth of large numbers of *Paramoeba* sp. 'AGD' was achieved by culture on malt yeast agar plates (Appendix 1.3) in 23cm² "Bioassay" dishes, as described in Method 2.1.3. For the first three culture challenges *Paramoeba* were grown with *S. maltophilia* (Appendix 2.1) as their food source, except in challenges 4 and 5 where *Paramoeba* were cultured with mixed normal flora from the gills (Appendix 2.3). *Paramoeba* cells were prepared by washing the surface of the agar plates with filter sterilised sea-water. No attempt was made to separate the amoebae from their bacterial food source.

The *Paramoeba* isolates used in the culture challenges varied between challenges, all were collected from AGD infected Atlantic salmon (see Table 4.1). The first three isolates used (PA-002, PA-013, PA-016) were collected during the studies conducted in Chapter 3. All other isolates were collected using the ammonium chloride wash, as described in method 2.2.1. All isolates were verified as *Paramoeba* species by the identification of one or more parasomes, using phase contrast microscopy (Method 2.3.1) and DAPI-staining (Method 2.3.2).

Table 4.1: *Paramoeba* isolate, isolation site, date of isolation and the challenge in which they were employed.

Isolate*	Age of isolates (months)**	Source	Challenge
PA-002	28	Nubeena	3
PA-013	26	Nubeena	3
PA-016	12/24	Dover	1 and 2
PA-021	19	Dover ²	3
PA-022	18	Dover ²	2
PA-024	18	Dover ²	2
PA-031	9/11	Dover	4 and 5
PA-010	5	Dover	5
PA-011	3/5	Dover	4 and 5
PA-018	3/5	Dover	4 and 5
PA-003	5	Dover	5
PA-009	3/5	Dover	4 and 5
PA-027	3/5	Dover	4 and 5
PA-028	3/5	Dover	4 and 5
PA-033	5	Dover	5

* Isolate number is not related to the date of isolation.

** Age of isolate at the beginning of each trial, where isolates were used in two trials, both ages are given.

1 Salmon Enterprises of Tasmania, marine farm, Dover

2 Tassal Pty Ltd, marine farm, Dover

Culture challenge 1

Rainbow trout were exposed to *Paramoeba* sp. 'AGD' with and without pre-conditioning of the gills. In an attempt to enhance susceptibility to infection by *Paramoeba*, trout were treated with benzylkonium chloride ('Zephiran') to cause a mild irritation to the gills. A total of 32 trout were bathed in 40 litres of sea-water containing 4 ppm of Zephiran for one hour at 16°C. Oxygen levels were kept low to encourage higher ventilation rates thereby increasing exposure to the irritant. Fish were marked by "Panjet" tattoo with alcian blue and allowed to recover overnight. Baseline gill samples from two Zephiran treated and two untreated trout were collected in Bouin's and sea-water Davidson's fixatives.

All Zephiran treated fish died soon after treatment. The remaining 20 trout, without preconditioned gills, were exposed to *Paramoeba* (PA-016) in a 20l bath of cell suspension for one hour at 16-18°C. The concentration of *Paramoeba* was estimated to be 9,000 amoebae/ml. Oxygenation rates were kept low to encourage higher ventilation rates across the gills and hence increase exposure to *Paramoeba*. Following exposure fish were transferred to a 2m² tank, and maintained in the flow-through tank system 1. Unexposed control trout were kept under the same conditions in a separate tank. Sea-water was heated to 18°C.

Fish were held for 14 days. Samples for histology were collected from two trout at day 7; gills, kidney, liver and spleen were collected in isotonic formalin; gill tissue was also collected in sea-water Davidson's fixative. Surviving fish were euthanased at the end of this period and gill samples collected in isotonic formalin and sea-water Davidson's fixatives. Control trout were sampled in the same manner. All fish were examined for the presence of mucous patches prior to being sampled.

Culture challenge 2

This challenge used the recirculation system described for tank system 2, in an attempt to achieve continued exposure of fish to cultured *Paramoeba* sp. ‘AGD’. Twenty five naïve Atlantic salmon were exposed to a mixture of three *Paramoeba* isolates (PA-016, PA-024 and PA-022), added to the tank approximately every three days, for 21 days. Figure 4.5 shows the day and cumulative number of *Paramoeba* added to the system. Water temperature was kept between 17 and 18°C. Baseline gill samples were collected in sea-water Davidson’s, for histological examination. Gill samples and gill smears were collected weekly from randomly selected salmon, or on a daily basis from moribund or recently dead salmon. After 29 days, all fish were euthanased, and gill samples and smears collected as before.

Culture challenge 3

This challenge was conducted as for culture challenge 2 using different *Paramoeba* isolates, in an attempt to expose fish to a wider range of *Paramoeba* isolates. Fourteen Atlantic salmon were exposed to a mixture of three *Paramoeba* isolates (PA-013, PA-002 and PA-021), for 33 days. The *Paramoeba* were added every 3-6 days (see Figure 4.5). The temperature of the water was kept at between 17 and 18°C. Gross gill pathology was observed weekly. Gill samples collected in sea-water Davidson’s fixative and gill smears were collected from six fish at the end of the challenge (day 33).

To determine the susceptibility of the fish to infection the remaining fish were incubated with fish suffering from AGD in the cohabitation challenge 2. These fish were sampled at 9 and 15 days post cohabitation.

Culture challenge 4

To increase the susceptibility of fish to infection with *Paramoeba*, photomanipulated 'out of season' Atlantic salmon were stressed prior to the addition of cultured *Paramoeba*. Fish were stressed in two ways: by injection with a cortico-steroid, methyl prednisolone (MPD), to suppress immune function; and by bathing with hydrogen peroxide to induce gill damage (Cameron, 1994).

Twenty fish were given intra-muscular injections of 0.17ml of MPD, representing a dose of 16mg/kg (Carson and Handler, 1988) the adipose fin of each salmon was clipped to designate treatment. Twenty fish were bathed for one hour in 50 litres of sea-water containing 300 ppm of hydrogen peroxide to induce gill damage, the anal fin of each fish was clipped to designate treatment. The stressed fish and 20 unstressed fish were placed into the recirculation tank for challenge with cultured *Paramoeba*.

The salmon were then exposed to a mixture of six *Paramoeba* isolates that represented more recently collected isolates (PA-031, PA-011, PA-018, PA-009, PA-027 and PA-028). *Paramoeba* were grown as previously described, except *S. maltophilia* as a bacterial food source was substituted with mixed normal bacterial flora from the gills of AGD infected salmon. To reduce the risk of *Flexibacter* infection occurring in the MPD stressed fish the *Paramoeba* were collected into sterile sea-water with antibiotics added at a ratio of 1:1000 (Appendices 3.1 and 3.2). *Paramoeba* was added two hours after the salmon had been placed into the tanks, to reduce the risk of residual hydrogen peroxide killing the *Paramoeba*, and then at intervals during the challenge period (see Figure 4.5). The temperature was maintained between 17 and 18°C. The duration of this challenge was 52 days.

Baseline gill samples were collected from the untreated and hydrogen peroxide stressed fish in sea-water Davidson's fixative, no baselines were obtained from cortico-steroid stressed fish. Gill samples were collected in sea-water Davidson's fixative at days 17, 21, 36 and at the end of the challenge at day 52. Gill smears were also obtained at day 36 and 52.

Culture challenge 5

Thirty Atlantic salmon were exposed to nine *Paramoeba* isolates (PA-028, PA-027, PA-018, PA-010, PA-011, PA-003, PA-009, PA-031 and PA-033), added to the system at intervals over the challenge period (see Figure 4.5). *Paramoeba* were grown with normal bacterial gill flora, as described for culture challenge 4. In this experiment the pump in the recirculation system failed, hence the fish were placed into the tank with 300 bioballs, acting as the biofilter. This system was static system with no recirculation, hence additional oxygen was supplied as described for tank system 1. Water was not heated during this experiment.

Gill samples (collected in sea-water Davidson's fixative) and gill smears were taken from two challenge and two control fish at days 1, 7, 20, and 26. Gill smears were also taken on day 14. At day 75 the challenge was terminated due to the sudden death of all the challenge fish, gill smears were taken from all dead salmon approximately 24 hours after death was thought to have occurred.

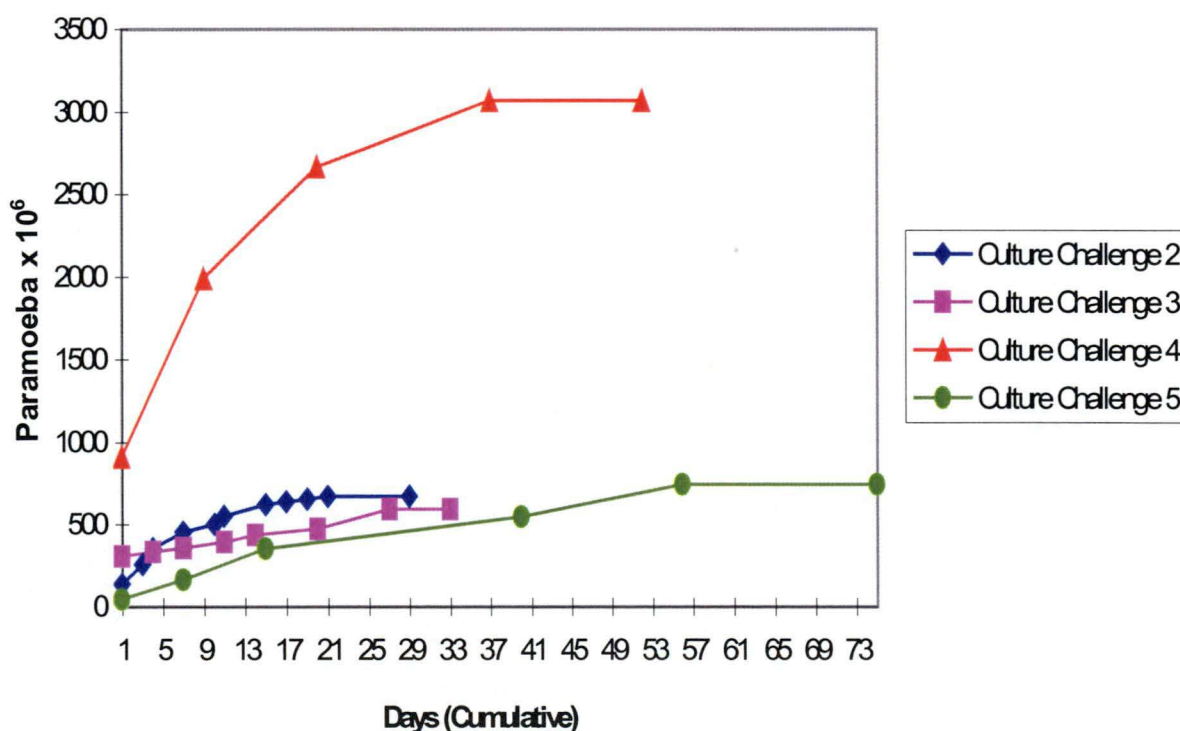


Figure 4.5: Cumulative number of cultured *Paramoeba* added to the tanks in each trial; each point indicates the addition of *Paramoeba* to the tank..

4.2.5 Cohabitation challenges

Cohabitation challenge 1

Twenty five naïve rainbow trout were placed into a separate tank of the flow-through tank system 1, with approximately 40 Atlantic salmon affected with severe AGD acquired naturally in sea-pens. Only Atlantic salmon with severe AGD (>10 patches) were selected for cohabitation. Baseline gill samples were collected from two of the naïve trout and two AGD affected Atlantic salmon, in Bouin's and sea-water Davidson's fixatives. The fish were held for 14 days at 18°C.

Samples for histology were collected from two trout and two AGD infected salmon at day 7; gills, kidney, liver and spleen were collected in isotonic formalin; gill tissue was also collected in sea-water Davidson's. Surviving fish were euthanased at the end of the challenge period and gill samples collected in isotonic formalin and sea-water Davidson's fixatives. The control fish for this experiment were as for culture challenge 1 and were sampled in the same manner.

Cohabitation challenge 2

Nineteen naïve Atlantic salmon were placed into a 1m² flow-through tank with three AGD infected fish. The AGD infected fish consisted of two with low level AGD (1-5 patches) and one moderately infected fish (4-6 patches). Approximately 50 control fish were kept in a separate tank, under the same conditions, without infected donor fish. Gross gill health of the challenge and control fish was scored periodically during the first 33 days. On day 33, gill samples (collected in sea-water Davidson's fixative) and smears were collected from four fish, from each tank. To determine the susceptibility of culture challenged fish to AGD, seven fish from the culture challenge 3 were added to the cohabitation tank at day 33. The challenge was terminated at day 48 and the remaining challenge fish euthanased, and gill samples and smears collected as on day 33. Control fish were also sampled as described.

Cohabitation challenge 3

Three infected Atlantic salmon, with low level infection (1-5 patches), were placed into a 1m² flow-through tank with approximately 40 naïve salmon. Regular checks of gross gill pathology were made throughout the duration of the challenge. Gill samples were collected in sea-water Davidson's fixative two months into the challenge. This challenge was undertaken to determine how long infection could be maintained in a tank system and to provide infected fish for isolation and for the production of positive gill smears (Appendix 8.1). Limited gill histopathology samples were taken to reduce unnecessary stress that may contribute to premature death of the infected fish. The aim of this challenge was to maintain infection for as long as possible. Approximately 80 control fish were held in a separate tank under the same conditions, without infected donor fish.

4.2.6 Electron microscopy

In an attempt to assess the virulence of cultured *Paramoeba*, three *Paramoeba* isolates (PA-002, PA-016 and PA-022) were examined by electron microscopy for the presence of hair-like filaments on the surface of the cells, as well as other ultramicroscopic features of the genus *Paramoeba*. The *Paramoeba* were prepared as described in Method 2.8; and examined in an a Hitachi H300 transmission electron microscope at 5,000X magnification to observe the structure of the parasome, and 40,000X magnification to observe the surface membrane.

4.3 RESULTS

4.3.1 Culture challenges

Culture challenge 1

Rainbow trout treated with Zephiran showed severe signs of stress after 20 minutes exposure and were moribund after 30 minutes. Gill histopathology from these fish showed marked epithelial lifting, variable to severe oedema, necrosis of the basal and chloride cells, congestion of the primary vessels and high levels of mucus. Untreated fish showed; occasional epithelial lifting, some prominent chloride cells, the occasional small basal nodule, but no obvious mucus and were otherwise normal. None of the Zephiran treated fish survived and were thus unable to be exposed to cultured *Paramoeba*.

Fish bathed in cultured *Paramoeba* showed no signs of distress during or after treatment, but a mortality rate of 50% occurred during the 14 days of the trial. Gross gill pathology was normal at each sampling. Gill histopathology of exposed fish was as normal, except for an increased number and prominence of chloride cells and slightly thickened tips. No amoebae or signs of AGD were observed in the gills. Liver, kidney and spleen samples were all normal.

The mortality level in the control tank was 42%. Gill histopathology at day 0 and 7 were normal as described for the untreated fish above; slight epithelial lifting indicative of heat stress was observed at day 14 but there was no evidence of AGD in any of the fish. Liver, kidney and spleen were normal on histological examination. The mean temperature of the tanks was 18°C, most of the mortalities occurring at temperatures between 18-21°C. To determine if vibriosis, caused by the bacterium *Vibrio anguillarum*, was causing the observed high mortality gill smears were taken at day 7. The results showed no significant bacterial findings.

Culture challenge 2

Gross gill pathology and gill histopathology of baseline fish showed no AGD or other significant gill changes, and gill smears showed no *Paramoeba* detected by IFAT. Smears taken at day 4 showed the occasional small fluorescent body, however these bodies were smaller than the *Paramoeba* usually observed in fish naturally infected with AGD. No evidence of AGD was observed in gill sections sampled on day 4. Gill histopathology at day 7 to day 22 showed patches of partial fusion of the secondary lamellae, some epithelial lifting and ballooning of the epithelium, some samples had high levels of mucus. No *Paramoeba* were observed in gill sections or in smears taken on these days. At day 29 the gill sections showed some thickening of primary tips and the occasional foci of fusion of adjacent pairs of secondary lamellae, and high numbers of chloride cells; but no areas with typical AGD pathological changes or *Paramoeba* were observed. Gill smears taken at day 29 were all negative for *Paramoeba*. Gross gill pathology was normal at each sampling. The mean temperature of the tank was 18°C. The overall mortality rate in this challenge was 40%.

Culture Challenge 3

Gross gill pathology and gill histopathology was normal in the baseline fish. Gill sections taken on day 33 showed no AGD-like pathological changes but gill pathology involving the fusion of the tips of the secondary lamellae, consistent with clubbed necrosis syndrome (see Chapter 3). Small fluorescent amoebae were observed in three of six smears taken on day 33, however these were smaller than *Paramoeba* usually observed in fish naturally infected with AGD. Gross gill pathology was also normal at day 33. The mortality of fish in this experiment was 14%. The mean temperature of the tank was 16°C.

Susceptibility of the exposed fish to AGD was confirmed by histology 9 days after the remaining fish were transferred to a tank with AGD infected fish (cohabitation challenge 2), gill changes consistent with AGD and clubbing and necrosis gill syndrome (CNG) were also observed in these fish. These fish showed large numbers of mucous patches on the gills, indicative of severe AGD. In addition large numbers of *Paramoeba* were detected in gill smears.

Culture challenge 4

Gross gill pathology was normal in the baseline fish. Gill histopathology was normal except for the presence of prominent chloride cells. Histopathology of fish treated with hydrogen peroxide showed extensive gill damage characterised by areas of basal vacuolation and linear separation of the epithelium, epithelial swelling, degeneration and necrosis. At day 17, gill histopathology of unstressed fish was normal, with less prominent chloride cells than baseline samples, and an increase in lymphoid cells. MPD treated fish showed evidence of deep lymphoid nodules, and one out of two hydrogen peroxide treated fish sampled showed considerable epithelial lifting. No evidence of AGD-like pathological changes were noted. At day 21 the gill histopathology of stressed and unstressed fish was similar to day 17, however one MPD treated fish showed two small lymphoid patches and one extensive AGD-like lymphoid patch but no *Paramoeba*. Gill histopathology at day 36 was again similar to previous samplings however one amoeba was observed in a hydrogen peroxide treated fish. Gill smears showed no evidence of *Paramoeba*, at day 17, 21 and 36.

At the termination of this challenge gill histopathology of five MPD treated fish showed some areas of fusion of the secondary lamellae thought to be AGD-like and a few associated amoebae; hydrogen peroxide treated fish showed variable changes with some small AGD-like patches of fusion but no amoebae. Untreated fish showed similar patches of fusion and several with floating amoebae. Testing of gill smears showed one untreated fish with the occasional *Paramoeba* (1-2 cells per field of view) and one hydrogen peroxide treated fish showed small numbers of amoebae (3-10 cells per field of view), the size of which were consistent with *Paramoeba* observed in smears from fish with naturally acquired AGD. Gross gill pathology was normal. The mean temperature of the tank was 16°C. The mortality of rate of this tank was 11.7%.

Culture challenge 5

Gross gill pathology and gill histopathology was normal at the commencement of this challenge. Gill histopathology at day 7 showed some epithelial lifting and thickening but no fusion of lamellae; however one fish showed very heavy congestion and large numbers of chloride cells but no amoebae. Gill smears were negative for *Paramoeba* by the IFAT. A dead fish sampled on day 11 was normal with none of the epithelial lifting observed at day 7. Gill smears taken at day 14 did not show *Paramoeba*. At day 20 and day 26, gill histopathology and gill smears of test fish showed no evidence of AGD or *Paramoeba*.

Periodic examination of the gross gill pathology of the challenge fish was made from day 26 to day 70. No significant mortalities or gross evidence of AGD were observed up to day 70. At day 75 the challenge fish suddenly died, the smell of the tank indicated the presence of hydrogen sulphide possibly as a result of back flushing from the waste pipe. Gill smears taken from the dead fish showed occasional to moderate numbers of *Paramoeba* (1-20 cells per field of view), these did not fluoresce as brightly as those in naturally acquired AGD and were smaller. Gill sections were not taken from the dead fish. The mean temperature of the tank was 17°C. The mortality rate in the tank up to day 70 was 5%.

4.3.2 Cohabitation challenges

Cohabitation challenge 1

Baseline gross gill pathology and gill histopathology were normal for both the control and challenged fish. Gross gill pathology of challenged fish at day 7 showed numerous mucous patches (> 10), indicative of severe AGD. Gill histopathology at day 7 showed well-developed, severe AGD pathological changes. This damage was characterised by multiple foci of fusion of the secondary lamellae and hyperplasia involving most primary lamellae; chloride cells were prominent; epithelial lifting was often marked especially between lamellae and surrounding the amoebae. Multiple lesions were observed in the sections representing the whole spectrum of the disease,

from early AGD to moderate and more severe AGD. Many *Paramoeba* were observed adhered to areas of fusion, and also to unfused secondary lamellae, the number of which were more numerous than seen in naturally infected fish. Liver, kidney and spleen were all normal. Gross gill pathology and histopathology of the control fish were normal at day 7.

At day 14 only one trout was remained. Gross gill pathology of the fish that died between day 7 and day 14 showed evidence of numerous mucous patches on the gills indicative of severe AGD. Gross gill pathology of the remaining fish at day 14 showed numerous mucous patches, (> 10), on the gills consistent with severe AGD; this was confirmed by gill histopathology that showed severe damage characteristic of AGD and many amoebae in the gill tissue. The mortality rate of the rainbow trout in this trial was 81%.

The mortality rate of the Atlantic salmon, the donor fish with naturally acquired AGD was 67%. Gill histopathology at day 0 and 14 showed severe AGD, characterised as described for the infected cohabited trout, although the number of amoebae in the gills was less than observed in cohabited fish. Overall the lesions observed were uniformly advanced. The control fish were as described for culture challenge 1, normal except for some heat stress and a mortality rate of 42%. The mean temperature of the test and control tanks was 18°C. The greatest number of mortalities occurred between 18 and 21°C.

Cohabitation challenge 2

Baseline gross gill pathology and gill histopathology was normal for both the control and challenged fish. At day 20, gross gill pathology of the challenged fish showed the presence of mucous patches on the gills consistent with mild to moderate disease. At day 26 all fish showed more than 10 mucous patches each, indicating severe AGD. Resolving patches were also noted. Gill histopathology of four fish at day 33 showed evidence of AGD pathological changes, and extensive patches of lamellar fusion with numerous *Paramoeba*. Gill smears taken at this time showed the presence of large numbers of *Paramoeba* (>20 cells per field of view).

Five mortalities occurred in the first 20 days of the trial, no more were recorded until after day 33. At the termination of the trial (day 48) only one cohabited fish remained, and five out of seven ex-culture challenge fish 15 days into cohabitation. Gill histopathology of the remaining fish showed evidence of severe AGD. Numerous *Paramoeba* were observed in gill smears (>20 cells per field of view). Control fish were normal at all samplings. The mean temperature of the tanks was 15°C. The mortality rate of the donor fish was 100% in this challenge and 95% for cohabited fish.

Cohabitation challenge 3

Baseline gross gill pathology and gill histopathology was normal for both the control and challenged fish. At day 28, examination of gross gill pathology showed the presence of small numbers of mucous patches indicative of early AGD. Gill smears taken from the fish showed the presence of small to moderate numbers of *Paramoeba* when tested by IFAT (3-20 cells per field of view). Periodic gross and histopathological gill examination showed that AGD was maintained at a low level. The mortality rate of this tank was low with few fish dying (number not recorded). AGD was maintained for the duration of the challenge, until the untimely death of all fish at day 158. The dead fish were not sampled due to death of the fish over the weekend. A strong smell of hydrogen sulphide was apparent in the tank indicating that death may be due to a tank system failure resulting from waste back-flush, as observed for culture challenge 5. Control fish were normal at all samplings. The mean temperature of the tanks was 13°C.

4.3.3 Electron microscopy

All three *Paramoeba* isolates showed a nucleus with a central nucleolus and the presence of a single parasome adjacent to the nucleus (Figure 4.6). All three isolates showed the presence of numerous hair-like filaments, and packed tubular elements on the outside surface of all three *Paramoeba* isolates tested (Figures 4.7 and 4.8). No scales were observed on the surface membrane of the isolates.

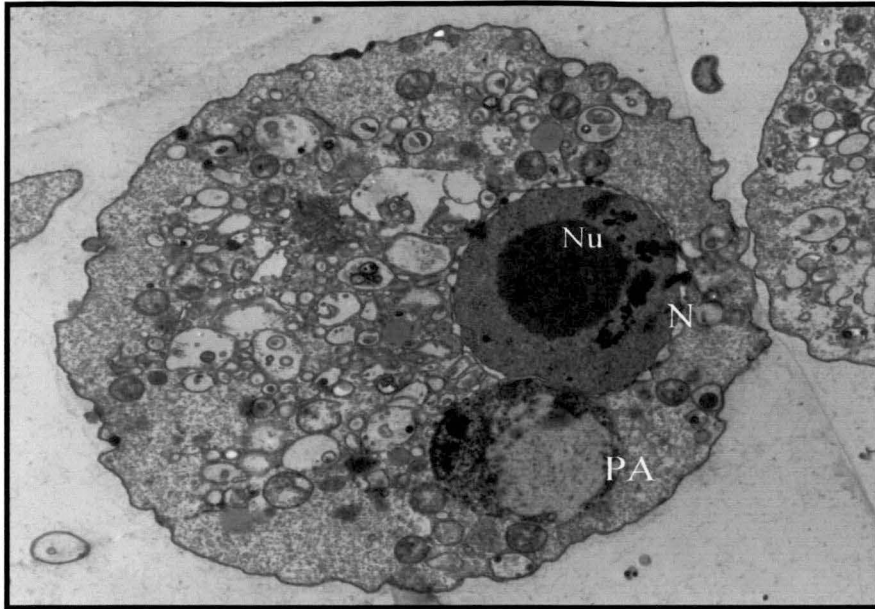


Figure 4.6: Cultured *Paramoeba* sp. 'AGD', electronmicrograph showing nucleus (N) with central nucleolus (Nu) and parasome (P) adjacent to the nucleus, X 5000

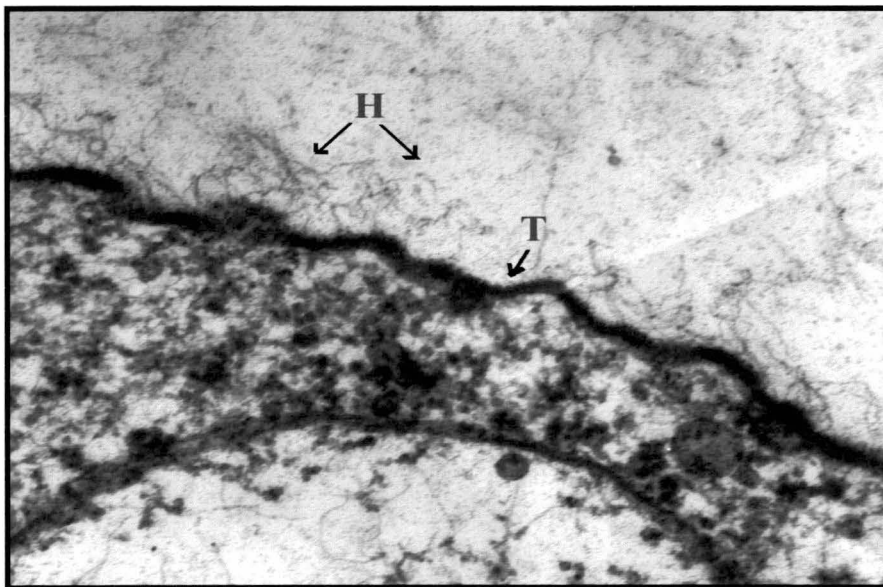


Figure 4.7: Cultured *Paramoeba* sp. 'AGD', electronmicrograph showing surface membrane with hair-like filaments (H) and tubular elements (T), X 40,000

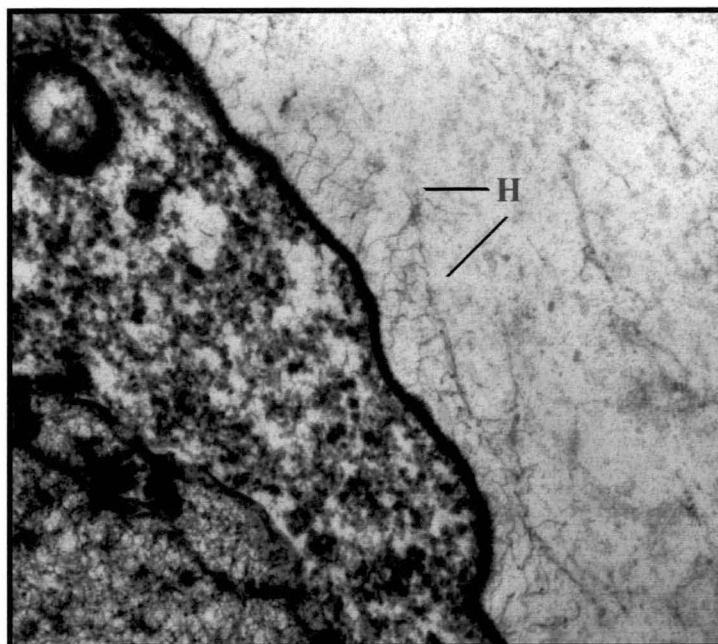


Figure 4.8: Cultured *Paramoeba* sp. 'AGD', electromicrograph showing surface membrane with hair-like filaments (H), X 40,000

4.4 DISCUSSION

Immunostaining of gill sections from AGD infected Atlantic salmon has verified that a *Paramoeba* species is associated with damaged gills, often in large numbers (Chapter 3), and excluded the overt involvement of other amoeba species isolated from damaged gills. Demonstration of the pathogenic role of *Paramoeba* is important for a number of reasons: it would fulfil Koch's postulates, and provide unequivocal proof that the cultured *Paramoeba* are virulent. If the cultured *Paramoeba* were proved virulent this could enable a model of infection to be developed using cultured amobae, allowing controlled study of AGD.

Attempts to infect susceptible fish species with AGD using cultured *Paramoeba* in the past have been unsuccessful (Jones, 1988; Kent *et al*, 1988; L. Searle, Pers. Comm.). A previous study showed that naïve fish could be infected with AGD when cohabited with AGD infected fish (L. Searle, Pers. Comm.). Infection of naïve fish with infected fish would not fulfil Koch's postulate but would provide a simple model of infection requiring naturally infected fish. For this method to be useful, infection would need to be defined, and controlled so that it could be maintained for extended periods without premature death of the infected fish.

A model of infection and maintenance of infection, by whichever means, would be an important tool to study the infection process. Study of the disease would then not be limited by the seasonality of the disease, providing AGD infected fish all year round, greatly increasing the knowledge of infection. In addition drugs, treatments and vaccines could be tested under controlled conditions.

There are numerous problems associated with keeping fish in artificial tank systems. The species of fish used, oxygen levels, water quality and temperature are all parameters that must be taken into consideration. After the first cohabitation and culture challenges the tank system was modified due to a number of actual and potential problems. A high mortality not associated with AGD was observed with this system, approaching 50% in the control and culture challenge fish. More importantly

the first system resulted in rapid infection and death in the cohabited fish. The high mortality of the fish in this system and rapidity of infection in cohabited fish was partially attributed to high temperatures, up to 21°C, when most of the mortalities occurred. Part of the strategy to slow down the progression of AGD in the cohabitation challenges was to keep temperatures in the tank lower than was experienced in the initial challenge. The flow-through part of the system was maintained in the modifications exclusively for the cohabitation challenges, sea-water supplied to these tanks was not heated.

The flow-through set-up of tank system 1 had a number of limitations for culture challenges. This system did not preclude the introduction of naturally occurring *Paramoeba* species, and more importantly exposure of the fish to cultured *Paramoeba* would be transient, and perhaps not long enough to allow colonisation of the fish gills. To extend the period of time that the fish were exposed to the *Paramoeba*, a recirculation system was developed. Water in this system was filtered to remove naturally occurring *Paramoeba*. The water was also heated to provide additional stress to the fish and to emulate the temperatures observed in the field when AGD is most prevalent. The biofilter employed in this system was extremely effective in keeping the level of toxic metabolites at a level where the fish remained healthy. The recirculation part of system 2 was used for all subsequent culture challenges except challenge 5 when the untimely demise of the one of the pumps forced the use of a static system, with biofilter balls placed in the water on top of the tank.

Susceptibility trials required the use of naïve fish with no natural exposure to *Paramoeba*. As acclimatisation of Atlantic salmon was thought to be less predictable outside the smolt window it was decided to undertake initial infectivity challenges using rainbow trout that can be adapted readily to sea-water throughout much of the year. Sea-caged rainbow trout are susceptible to AGD, although the level of susceptibility compared to Atlantic salmon is not known. However, in this study the high mortality of the control rainbow trout in the first challenges suggested that rainbow trout might be unsuitable for future challenge experiments. This finding was

perhaps to be predicted as rainbow trout are no longer farmed in full sea-water in Tasmania due to high mortalities, most probably as a result of high water temperatures combined with full salinity, and thus they are now only farmed in brackish water sites (Purser, 1992; McKelvie *et al*, 1994). High temperatures combined with full salinity were thought to be the reason for the high mortalities observed in the first cohabitation and culture trials. Subsequently all further challenges were undertaken with Atlantic salmon smolt. The salmon were not acclimatised to sea-water but were fed on a salt supplemented diet prior to placement in sea-water to assist in preparing them for full sea-water, a recent process that has now replaced the slow acclimatisation to sea-water previously used by farms to prepare smolt for the sea (S. Oddsson, Pers. Comm.).

A change in the fish species, combined with modifications to the tank system, was thought to be the main reasons why the mortality rate of fish in the culture challenges was reduced from 50% in the first challenge to around 5% in culture challenge 5. Benefits were also observed in the cohabitation experiments where infection progressed more slowly and was maintained for longer periods.

4.4.1 Culture challenges

Infection of naïve fish with AGD using cultured *Paramoeba* sp. ‘AGD’ was not convincingly demonstrated in this study. There was some evidence of colonisation of amoebae, as indicated by the presence of *Paramoeba* in some gill smears tested by the IFAT, and in some gill sections the presence of AGD-like pathological changes in which small numbers of *Paramoeba* were observed. However, there was no gross evidence of AGD, indicated by the absence of mucous patches on the gills of the fish, and no mortalities could be attributed to AGD. Despite some promising results, cultured *Paramoeba* could not be considered pathogenic under the conditions employed. However, the results of this study provide some interesting information regarding the process and factors affecting experimental transmission of *Paramoeba* sp. ‘AGD’ in fish.

One possible explanation for the failure of these challenges may be that the *Paramoeba* had lost virulence in culture, or were not virulent using the culture and challenge conditions employed. Loss of virulence in cultured amoebae has been described (Jellet and Scheibling, 1988b; Gupta *et al*, 1998). Jellet and Scheibling (1988b) reported a loss in virulence of *Paramoeba invadens* (the causative agent of paramoebiasis in sea urchins) after 15 weeks in monoaxenic culture and 58 weeks in polyaxenic culture. They suggested long term maintenance of *P. invadens* in polyaxenic culture over monoaxenic culture. Periodic passage through the host was also suggested as a means to improve or restore virulence. A gradual loss of virulence in culture has also been described in *Entamoeba histolytica* maintained in polyaxenic culture, which was restored by passage through host cells (Vincent and Neal, 1960 as cited in Jellet and Scheibling, 1988b). Gupta *et al* (1998) reported similar results restoring the virulence of *E. histolytica* by passage in hamster liver. Passage of *Paramoeba* sp. 'AGD' in its marine host could not be attempted due to the obvious inability to experimentally transmit the disease. A possible culture technique not investigated is the culture of *Paramoeba* in cell culture, using fish cells. Bols *et al* (1994) reported the development of a cell line derived from the gills of rainbow trout, and suggested the use of this cell line for the study of gill diseases in fish, this cell line may also be useful in the culture of virulent organisms.

Apart from the culture conditions affecting the virulence of *P. invadens*, other factors affecting virulence of *Paramoeba* species have not been extensively studied. Detailed studies of the virulence of *Paramoeba* species in marine hosts have not been reported. As *Paramoeba* sp. 'AGD' are unable to be preserved at -70°C (Chapter 3), and large numbers were required for culture challenges, there were limited ways to protect against the *Paramoeba* becoming laboratory adapted and losing virulence.

A number of methods were employed to improve the chances of exposing the fish to a virulent *Paramoeba* isolate. When AGD did not occur in the first challenge using one *Paramoeba* isolate, successive challenges using between three and nine different isolates representing a total of 15 were tried, in the hope that one or more isolates may have retained virulence in culture. The first three culture challenges used *Paramoeba* isolates that were in excess of 12 months old. Fresh isolations were conducted and in the remaining two challenges the fish were exposed to younger cultures, on average between three and five months old at the start of the challenge. To further enhance or restore virulence these fresher isolates were cultured on a mixed bacterial substrate grown from bacterial colonies accompanying primary isolations of *Paramoeba*. Although these new isolates failed to produce typical AGD in exposed fish, the last two challenges were the only ones where some gill smears were found positive for *Paramoeba*, and where there was some evidence of AGD-like pathological changes in the gills, with a few associated amoebae. It was interesting that in culture challenge 4 several gill sections showed the presence of floating amoebae, not attached to areas of abnormal pathology as expected, which may suggest that the cultured *Paramoeba* had reduced ability to attach to the gill tissue. Further evidence of this came from the results of culture challenge 5 where evidence of infection was only seen in dead fish, the gill smears showing occasional to moderate numbers of *Paramoeba*, indicative of slight to moderate AGD in the field (see Chapter 6), which suggests that the *Paramoeba* could only attach to the gills after death. In the culture challenges undertaken by Jones (1988), *Paramoeba* was isolated from the sediments and on twine attached to challenged fish but not in the gills, suggesting that they survived the challenge process but were unable to attach to the gills and cause infection. Collier *et al*, (1985) described an avirulent strain of *Mycoplasma pneumoniae* in which loss of virulence was characterised by a loss of the organisms' ability to attach to the respiratory epithelium. The authors suggested this loss of virulence might be due to mutational events altering the known binding surface protein responsible for attachment. Custodio *et al* (1995) demonstrated that *Neoparamoeba aestuarina* (formerly *Paramoeba aestuarina*) lost their ability to attach to the bottom of culture dishes when grown in culture medium containing a specific heptapeptide. A loss of attachment ability could have occurred in the cultured *Paramoeba* sp. 'AGD'. Studies

to determine virulence of the cultured *Paramoeba* compared to gill attached organisms are warranted. Significant work has been done on the virulence of the human amoeboid pathogen *Entamoeba histolytica*, especially in relationship to culture conditions, using isoenzyme (zymodeme) patterns to identify pathogenic markers (Mirelman *et al*, 1986; Mirelman, 1987; Gitler and Mirelman, 1987; Mirelman and Chayen, 1990; Mills and Golsmid, 1995). Similar studies may elucidate virulence markers in *Paramoeba* sp. 'AGD'.

To further improve transmission, the fish were exposed to a much greater number of *Paramoeba* than is believed to occur in the natural environment. Jones (1988), found only 3.4 *Paramoeba* per litre of sea-water, sampled from a sea-cage with high mortalities due to AGD, while none were found in samples taken from mildly affected cages. However the numbers found on the gills of AGD affected fish were as high as 4.9×10^6 . Rogerson and Laybourn (1992) reported an annual mean of 8300 amoebae per litre in an estuary in Scotland and an occasional summer maximum of 43000 per litre. In this study large numbers of cultured *Paramoeba* were cumulatively added to the tank system, the smallest cumulative number added equated to approximately 0.27×10^6 per litre and the largest 1.39×10^6 per litre, the numbers being far in excess of the number of free-living amoebae thought to occur in the marine environment.¹ It is thus unlikely that the numbers of *Paramoeba* added were too low to cause infection.

Paramoeba appear to rapidly proliferate on the gills of fish. Perhaps the *Paramoeba* colonise an intermediary host or surface before colonising the gills of the fish, such as macroalgae or nets. *Paramoeba* is one of the most common amoebae found on species of macroalgae (Rogerson, 1991). Rogerson reported that amoeba species on two types of macroalgae were most abundant in the summer months, where numbers up to 23 amoebae per cm² of algal surface were recorded. *Paramoeba* has been

¹ Zilberg *et al* (2001) found that the lowest concentration of *Paramoeba* sp. (harvested from naturally infected fish) required to cause AGD was 230 *Paramoeba* per litre. This paper was published after submission of this thesis and was not extensively reviewed in this study.

observed on the nets of sea-cages containing AGD infected salmon (Jones, 1988). It is also known that *Paramoeba* will not grow or multiply in suspension but require a solid substrate for *Paramoeba* growth (Martin, 1985). *Paramoeba pemaquidensis* has been reported in marine sediments off the Western North Atlantic, the Bight of New York and the Gulf of Mexico (Sawyer, 1980). Anderson (1998) also reported the abundance and diversity of marine gymnamoebae in sediments. Recently, Douglas-Helders (2000) established that *Paramoeba* from dead AGD infected fish could colonise the gills of previously uninfected dead fish, indicating that AGD mortalities present in sea-cages might be an important reservoir of infection. Although it is not known if healthy naïve fish can be infected with *Paramoeba* from dead fish. The ability of the cultured *Paramoeba* to colonise the tank surface in these challenge experiments is not known. The absence of a suitable solid substrate in the tanks, such as nets, macroalgae, sediments and dead fish are factors that might have affected these challenges. Further work is required to elucidate if these solid substrates are potential reservoirs of infection in the field. The IFAT (as discussed in Chapter 6) may be a simple method to determine if *Paramoeba* are present on the surface of macroalgae, nets and perhaps sediments; and has already been used in the study of dead fish by Douglas-Helders *et al* (2000)

The duration of the exposure to *Paramoeba* was increased with each subsequent challenge, the last challenge lasting for 75 days. The duration of the challenges was thought to be adequate as infection in sea-cages can be rapid, sometimes occurring 6 weeks post transfer to the sea (S. Oddsson, Pers. Comm.). With elevated temperatures, stress to the fish and the large numbers of *Paramoeba* added to the tanks, AGD was expected to occur at a much faster rate than occurs in the sea-cages. Cohabitation experiments showed that infection in the tanks could be rapid. In addition, the culture challenged fish were found to be highly susceptible to AGD when cohabited with infected fish. It is therefore unlikely that the duration of the challenges was too short for infection to occur.

It appears that a common feature of all diseases caused by *Paramoeba* species is some form of stress predisposing disease. *P. perniciosus*, the causative agent of paramoebiasis in crabs, is thought to enter the soft cuticles of the crabs during the summer molting period, the peak time for this disease. Heightened susceptibility of sea urchins in high sea-water temperatures, when the host's resistance is low, has also been postulated as the predisposing factor for the infection with *P. invadens* (Jones and Scheibling, 1985). Past researchers have suggested that *Paramoeba* is likely to be an opportunistic pathogen that causes disease under certain conditions such as mechanical, chemical or parasitic damage; bacterial load of the gills has also been implicated (Kent *et al*, 1988; Roubal *et al*, 1989; Munday *et al*, 1990, Cameron, 1993). In the sequential gill health study undertaken in Chapter 3 damage was observed in the gills before the colonisation of *Paramoeba*. Cameron (1993), observed a progressive development of nodules and plaques in the gill tissue which were preferentially colonised by *Paramoeba*, these plaques only being associated with the marine environment and were not pre-existing from the freshwater phase of life. These results have been confirmed in subsequent studies (Nowak and Munday, 1994; Clark and Nowak, 1998).

With these factors in mind, a number of methods were employed in this study to render fish more susceptible to infection and subsequent development of the disease: gill irritation with Zephiran and hydrogen peroxide; and immunosuppression using MPD. Increasing the water temperature in the tanks was also used to stress the fish and provide water temperatures that favour the development of AGD in the field.

In culture challenge 1, Zephiran (benzyolkonium chloride) was used to irritate the gills. Zephiran has been shown to irritate the gills of rainbow trout and chinook salmon exposed to a concentrations of 3 ppm in freshwater for one hour (Hoskins and Dalziel, 1984; Byrne *et al.*, 1989); and in Atlantic salmon smolt exposed to a concentration of 2 ppm in sea-water for three hours (Jones, 1988), without causing death. An exposure of 4 ppm in sea-water for one hour was attempted, however it was not successful in causing irritation without death. Levels above 4 ppm in freshwater have been reported to cause death in rainbow trout (Hoskins and Dalziel, 1984). Hence in hindsight it was not surprising that the fish exposed in this challenge did not survive.

In culture challenge 4, hydrogen peroxide at 300 ppm for 1 hour was used to irritate the gills of fish. Hydrogen peroxide has been found to be toxic to salmon smolt at concentrations of 300 ppm or greater when exposed for 3 hours at 12°C, however at one hour exposure considerable gill damage is induced without death (Cameron, 1994a). After exposure the gills showed extensive damage and necrosis that had partially healed by day 17, when only epithelial lifting was noted. Methyl prednisolone was used to suppress immune function in an attempt to render the fish more susceptible to AGD. Methyl prednisolone (MPD) is a glucocorticoid that suppresses immune function, it has a potency five times that of cortisol² (J. Carson, Pers. Comm.) The use of cortisol to suppress immune function and render fish more susceptible to experimental infection is well recognised (Johnson and Albright, 1992; Steinhagen *et al*, 1998). The level of MPD used in this study had previously been used to render the common goldfish more susceptible to a variant strain of *Aeromonas salmonicida* in transmission studies (Carson and Handler, 1988). Lindenstrøm and Buchmann (1998) found MPD (at 30mg/kg) increased the susceptibility of rainbow trout to infection with the parasitising skin fluke *Gyrodactylus derjavini*, although the reasons for this increased susceptibility were unknown. No initial damage or reaction to the gills was expected after MPD inoculation, however by day 17 the gills showed evidence of deep lymphoid nodules; it was hoped the *Paramoeba* would preferentially colonise these nodules as previously described (Cameron, 1993a; Nowak and Munday, 1994; Clark and Nowak, 1998). Interestingly this challenge was the only challenge where evidence of *Paramoeba* colonisation was observed in gill sections and in gill smears. There was evidence of AGD-like histopathology in the gills of MPD stressed fish 25 days into the challenge. By day 56, areas of AGD-like pathological changes, some with a few amoebae, were observed in both unstressed and stressed fish. Failure to infect MPD and hydrogen peroxide stressed fish with typical AGD does not preclude pre-existing gill damage as a pre-disposing factor for AGD, the failure most likely being due to a loss of virulence in the cultured *Paramoeba* (as discussed). However, the results of this study have shown that gill

² A search of the literature has failed to find reference to the potency of MPD vs cortisol. This information was obtained by Dr Carson from a pharmacist at Launceston General Hospital, Tasmania.

damage can be successfully induced with hydrogen peroxide, and that MPD causes the development of deep lymphoid nodules in the gill that may serve as preferential sites for *Paramoeba* colonisation. The study conducted by Lindenstrøm and Buchmann (1998) inoculated fish with 30mg/kg weight of the fish, whereas in the present study 16mg/kg was used, thus the use of a higher concentration of MPD in future studies may be justified.

Challenge 4 had a number of unique parameters that may have resulted in the successful colonisation of the gills with *Paramoeba*, albeit limited colonisation. Aside from gill stressing this challenge used young *Paramoeba* cultures grown on normal bacterial gill flora, and the numbers were also larger than used in any other challenge. This result indicates that although stressing the fish may have rendered the fish more susceptible initially all groups were equally susceptible by the end of the challenge. The sheer numbers of *Paramoeba* added to the challenge and a likely increase in the numbers of virulent *Paramoeba* present in the system are the most likely reason for the observed colonisation. Despite colonisation no gross gill pathology indicative of AGD were noted and no mortalities were observed as a result of AGD.

It was not known if the limited colonisation observed in challenge 4 would have developed into AGD if the challenge had been continued. However challenge 5 was continued for 75 days and also used young *Paramoeba* cultures grown on normal bacterial gill flora, without overt signs of AGD. Failure of the cultured *Paramoeba* to colonise the gills in challenge 5 is not known, however a build-up of toxic metabolites were thought to have caused the death of the fish in the tank, and perhaps reduced the virulence of the cultured *Paramoeba*. The high numbers of positive IFAT smears and the density of amoebae observed in the dead fish sampled in challenge 5 suggests that the cultured organisms were only able to effectively colonise the gills after death. Perhaps the cultured *Paramoeba* were only able to attach to gills when water was not flowing over them.

Fluorescent amoebae were observed in a number of gill smears taken during challenges 2 to 5. The number of positive gill smears was small, except in challenge 5 where nearly all the smears taken from dead fish showed occasional to moderate numbers of fluorescent amoebae. In all smears except those observed in challenge 4 the amoebae were smaller than seen in smears from naturally infected fish. In challenge 4 only two positive smears were observed, however these amoebae were of similar size to naturally occurring *Paramoeba* observed in gill smears. The size of cultured *Paramoeba* has been reported as significantly smaller than gill attached *Paramoeba* (Kent *et al*, 1988; Roubal *et al*, 1989, and Munday *et al*, 1990), which may explain these findings. Page (1983) also reported that many species of marine amoebae are smaller in culture than those occurring naturally. Other researchers have reported that the size of marine amoebae is highly dependant on culture conditions and intensity of infection (Sawyer, 1969; Cann and Page, 1982). The occurrence of amoebae the same size as *Paramoeba* in naturally infected fish in challenge 4 may also explain the relative success of this challenge compared to the others.

The only clue in the literature to a possible virulence factor was the reported absence of surface hairs in cultured *Paramoeba*, a feature of gill-attached organisms (Kent *et al*, 1988; Roubal *et al*, 1989). Three older *Paramoeba* isolates that had been maintained in culture (monoaxenic) for up to two years were examined by electron microscopy for the presence of surface hairs. All three isolates showed the presence of fine surface hairs, a feature until now only observed in gill-attached organisms. The presence of parasomes, packed tubular elements in the outer membrane and surface hairs suggests that morphologically these isolates are most likely *Paramoeba pemaquidensis*, as described by Page (1983). No surface scales were observed on the outer membrane, excluding classification of these isolates as *Paramoeba eilhardi*. The observation of surface hairs in cultured isolates in this study is most probably a result of a more supportive fixation technique, whereby the *Paramoeba* were processed within agar blocks, rather than as a concentration of unsupported cells as employed by other researchers (Kent *et al*, 1988; Roubal *et al*, 1989). The effects that isolation method and culture conditions may have had on the development of surface hairs cannot be totally ruled out. However, based on these observations it is unlikely

that these hairs play an important role in pathogenicity as the same isolates failed to produce disease in the challenge trials. However, it is not known if the hair-like filaments were compromised during culture. If they are involved in attachment, and attachment is important for pathogenicity, then is a role for these hairs. Further study is required to determine if there are any differences between the hair-like filaments on gill attached *Paramoeba* and cultured *Paramoeba*.

The possibility that the isolates cultured from the gills of the fish in this study was not the *Paramoeba* species responsible for disease was largely discounted by immunostaining of AGD infected gill tissue with *Paramoeba* antiserum (Chapter 3). The presence of surface hairs on these isolates provides additional evidence that the isolates used are of the same species seen in fixed gill sections. However species specific testing of antiserum made from a different *Paramoeba* isolate (PA-027) collected during this study (see Chapter 3) has shown that it reacts to a number of related *Paramoeba* species including *P. pemaquidensis* (Douglas-Helders *et al*, 2001). Recent studies have compared some of the isolates to reference strains of *P. pemaquidensis*, two from AGD infected salmonids in other countries, and found 98% sequence similarity confirming these isolates as *P. pemaquidensis* (F. Wong, N. Elliot, J. Carson, Pers. Comm.). Therefore it is highly unlikely that the *Paramoeba* isolates used in the present study are not the species responsible for AGD.

4.4.2 Cohabitation challenges

Infection of naïve fish by cohabitation with infected fish is a well established method used to study bacterial and viral fish diseases (Enger *et al*, 1992; Nylund *et al*, 1994). The results of the first cohabitation challenge using naïve rainbow trout demonstrated that AGD could be reproduced in an artificial tank system. As no signs of AGD were seen in the control fish, the development of AGD could only be attributed to cohabitation. In this trial, development of AGD was extremely rapid and overwhelming, resulting in the death of nearly all fish within 14 days. The rapidity of infection and death was most likely a combination of elevated temperatures, increased susceptibility of rainbow trout to both AGD and temperature stress, the ratio of AGD

infected Atlantic salmon to naïve fish, and the severity of AGD in the infected fish. Death in the naturally infected Atlantic salmon was expected to be rapid, due to the severity of AGD in these fish, and the stress involved in the transfer to the artificial tank system. Interestingly the opposite was observed, the mortality rate in these fish being lower (67%) than in the rainbow trout (81%). This could be due to an increased tolerance of Atlantic salmon to AGD compared to rainbow trout, the size of the salmon, or perhaps evidence that these fish had developed some form of immunity that allowed them to limit the disease for longer than the trout.

For cohabitation infection to be used as a simple model of infection the fish would need to be maintained for long periods without death. To reduce the level and rapidity of infection, and to maintain infection for longer periods, subsequent challenges were undertaken using naïve Atlantic salmon, a reduced ratio of infected to naïve fish, and AGD infected fish with milder infection. In challenge 2, a reduced ratio of one infected fish to eight naïve fish, and infected fish with low to moderate level of infection, resulted in the first signs of AGD at day 20. Amoebic gill disease was maintained for 48 days in this challenge, by which time 95% of the fish had died. The last challenge used an even lower ratio of 1 infected fish to 13 naïve fish, with a lower level of infection. The water temperatures were also significantly lower than in the first trial, in challenge 3 a mean of 13°C was recorded. A combination of low ratio and infection, and lower temperatures resulted in infection being maintained for 158 days. It is clear from this study that AGD can be maintained for long periods in an artificial tank system. Stringent tank husbandry and the introduction of naïve fish to replace dead fish could allow AGD to be maintained in an artificial tank system indefinitely.

The results of these challenges suggest that *Paramoeba* can be effectively transmitted from host to host without the presence of parasitic isopods, as previously implicated (Chapter 3), although the contribution of these organisms in the development and severity of AGD in the field still remains to be elucidated. Under optimal conditions, such as a low ratio of infected to naïve fish (<1:8), and low water temperatures (<15°C), Atlantic salmon could be rapidly infected with AGD in cohabitation

challenges. Baseline gill samples of these fish showed little obvious gill damage suggesting that gill damage is not always required to establish infection. This is in agreement with the findings by Zilberg *et al* (2001), who reported the presence of *Paramoeba* sp. attached to normal gill tissue. However the development of gill damage in this study could have been rapid, occurring between sample times and have been missed. It is also unlikely that the challenge fish had enough time to establish high levels of marine bacterial flora that would precipitate disease, as theorised by Cameron (1993). However, the tank method of infection is artificial and may not truly represent the disease process in sea-cages.

From these challenges it appears that only a small number of infected fish are required to precipitate AGD in naïve fish. Perhaps only a small number of fish are initially colonised in the sea-cages, for reasons unknown, which then leads to infection in all susceptible fish. The rapid development of AGD in cohabitation challenges supports field observations where cages of infected fish act as a reservoir of infection for uninfected cages (S. Percival, Pers. Comm.). Jones (1988) observed *Paramoeba* species in a number of native fish species found in and adjacent to salmon cages, with one species showing severe gill damage with AGD-like pathological changes. These results may warrant the investigation of native fish in transmitting and maintaining AGD. In addition, the suggestion that dead fish may act as a reservoir of infection (Douglas-Helders *et al*, 2000) requires confirmation in tank trials.

4.5 CONCLUSIONS

Despite evidence that *Paramoeba* is the cause of AGD, and the numerous methods used to improve the likelihood of infection, Koch's postulates could not be fulfilled. All evidence suggests that the cultured *Paramoeba* used in these challenges was not virulent under the conditions employed. Only with young cultures grown on normal gill flora was there any evidence of colonisation of the gills. Colonisation of the gills was greatest in challenge 5 where all dead fish sampled showed occasional to moderate numbers of *Paramoeba* in gill smears, suggesting that the cultured organisms may not be able to attach to the gills of live fish. This finding suggests that

there may be more appropriate culture conditions that maintain the virulence of *Paramoeba*. Propagation of a virulent *Paramoeba*, able to attach and infect naïve fish, is one of the most important goals for AGD research.

The presence of hair-like filaments on the surface of cultured *Paramoeba* constitutes the first report of these structures in cultured *Paramoeba* from the gills of fish infected with AGD. The observation of surface hairs in cultured organisms in this study is probably a result of a more supportive fixation technique, whereby *Paramoeba* were processed within agar blocks. The presence of these hair-like filaments on cultured *Paramoeba* can be interpreted in two ways: either they do not play an important role in pathogenicity, as the same organisms failed to produce disease in challenge trials; or that they may have been compromised during culture. If they are involved in attachment, and attachment is important for pathogenicity, then there is a role for these hairs. Further study is required to determine if there are any differences between the hair-like filaments on gill attached *Paramoeba* and cultured *Paramoeba*.

The cohabitation model of infection was refined, allowing maintenance of disease for approximately 158 days. The results suggest that AGD could be maintained indefinitely in an artificial tank system, provided stringent husbandry practices are followed, and dead fish are replaced with naïve fish. This model of infection now allows controlled study of the disease process, providing researchers with a constant supply of fish to study without the seasonal constraints that have impeded research in the past. This simple model of infection can also be used to test potential chemotherapeutants and vaccines. Subsequent work in Tasmania has shown that this model of infection, when controlled, is a reliable method to study the disease process in AGD infected Atlantic salmon (Findlay *et al*, 1995 and 1998).

Chapter 5. Identification and screening of potential amoebicides

5.1 INTRODUCTION

In Tasmania, amoebic gill disease (AGD) is controlled by bathing the fish in freshwater for a period of 1 to 8 hours, and in some cases towing the pens to areas of brackish water (Munday *et al*, 1990). The therapeutic effects of freshwater are not fully understood. It is thought that freshwater bathing may work in a number of ways: reducing the number of *Paramoeba* on the gills; removing the mucus covering the gills; and temporarily reducing hypernatraemia (Munday *et al*, 1990). Visually during freshwater bathing, mucus is removed from the gills forming a scum on the surface of the sea (J. Smith, Pers. Comm.). It is not known whether freshwater is lethal to the *Paramoeba* species responsible for AGD.

Although freshwater bathing is effective, it is highly labour intensive and therefore expensive. It requires adequate supplies of freshwater, which places limitations on the sites that can be exploited for Atlantic salmon sea-farming in Tasmania (Clark and Nowak, 1999). It also exposes fish to potential damage or stress, and the risk of secondary opportunistic bacterial infections. Bath treatments with formalin, potassium permanganate, chelated copper, malachite green and chloramine-T, have all been ineffective in treating AGD (Munday *et al*, 1993). These chemical bath treatments are commonly used for the treatment of a wide spectrum of external parasites, including protozoa, in a variety of cultured fish species (Sedgewick, 1988).

While freshwater bathing is successful there may be other more effective and economic means based on medicated baths or feeds. Field trials of potential chemicals are costly and time consuming, and thus not an effective means of screening chemicals. In this study two methods were developed to screen chemicals for their amoebicidal activity to *Paramoeba in vitro*. The results of which were used to identify chemicals for field testing as feed or bath medications to treat AGD. In addition the effect of freshwater on *Paramoeba* survival was investigated.

5.2 METHODS

5.2.1 The isolate

The *Paramoeba* isolate used in this study was collected from an AGD infected Atlantic salmon, sampled at Dover in March 1992 (PA-016). The culture was maintained on malt yeast agar (Appendix 1.3) seeded with the bacterium *S. maltophilia* (Appendix 2.1) as a growth substrate, and subcultured as described in Method 2.1.2.

5.2.2 Chemicals

Chemicals and treatments with potential amoebicidal activity to *Paramoeba* sp. ‘AGD’ were selected from a wide range of anti-protozoan and anti-parasitic chemicals used in human and veterinary therapy; and from a range of chemicals and treatments used to treat fish diseases in the fish farming industry. The selected chemicals and treatments can be divided broadly into the following groups:

1. Anti-amoebic drugs used in the treatment of amoebic dysentery and hepatic amoebiasis in humans.
2. Anti-malarial drugs used in the treatment of malaria in humans.
3. Anti-coccidials used in controlling coccidiosis in poultry and pigs.
4. Anthelmintics used in controlling nematodes in a range of animals.
5. Chemicals used in the treatment of a range of parasites infecting cultured fish species.
6. Assorted chemicals and treatments used to treat protozoan and parasitic diseases of a variety of animals.

Table 5.1 lists the chemicals tested in this study. A brief description of each chemical or treatment investigated in this study is given in Appendix 14.

Table 5.1: List of potential amoebicidal chemicals and treatments tested by the growth inhibition and contact inhibition assays.

Chemical Name	Growth	Contact
Amprolium hydrochloride	X	
Berberine hydrochloride	X	
Zephiran (benzylkonium chloride)		X
Carnidazole	X	
5-Chloro-7-iodohydroxyquinoline	X	X
Chloramine-T		X
Chloroquine diphosphate	X	
Diloxanide furoate	X	
Emetine hydrochloride	X	
Ethanol	X	X
Freshwater		X
Fumagillin	X	
Hydrogen peroxide		X
2-Hydroxy-1,4 naphthoquinone	X	
8-Hydroxyquinoline	X	X
Ivermectin	X	
Juglone (5-Hydroxy-1,4 naphthoquinone)	X	
Levamisole	X	
Mebendazole	X	
Mefloquine hydrochloride	X	
Metronidazole	X	
Monensin	X	
Naphthalophos	X	
Narasin	X	X
Nifurpirinol	X	
Niridazole	X	
Nitrothiazole	X	
Nitrofurantoin	X	
4-Nitroimidazole	X	
Oxfendazole	X	
Ozone		X
Phthalylsulphathiazole	X	
Praziquantel	X	
Primaquine diphosphate	X	
Pyrantel	X	
Pyrimethamine	X	X
Quinacrine hydrochloride	X	X
Quinine hydrochloride	X	
Quinoline	X	X
Sea-water pH=6		X
Sulphaquinoxaline	X	
Tinidazole	X	
Toltrazuril	X	

All the chemicals were solubilised using recommended solvents (The Merck Index. 11th edition, 1989); and filter sterilised as described in Method 2.9.1. Water, ethanol, and sodium hydroxide were the solvents used in this study. Acetone and dimethyl formamide were found to be inhibitory to *Paramoeba* sp. 'AGD' and were not used. Fumagillin and mebendazole, were not able to be solubilised as the recommended solvents inhibited the growth of *Paramoeba*, both were used as suspensions.

5.2.3 Chemical screening methods

Growth inhibition assay

This method was used to determine the ability of *Paramoeba* sp. 'AGD' to grow and/or survive in the presence of selected chemicals, and to determine the minimum inhibitory concentration (MIC). Potential anti-amoebic chemicals were incorporated into non-nutrient agar at concentrations between 5-30µg/ml. The test agar was surface seeded with nutrient bacteria and inoculated with varying dilutions of *Paramoeba* sp. 'AGD'. Growth was scored after 2 weeks and compared to control plates containing no chemical. The minimum inhibitory concentration (MIC) was determined as the lowest concentration able to inhibit growth, and these results of which were used to identify chemicals that could be used in feed to treat fish infected with amoebic gill disease and to select which chemicals were to be tested by the contact inhibition assay. For a more detailed methodology see Method 2.9.2.

Contact inhibition assay

This method was developed to determine the amoebicidal activity of a compound or treatment in a short-term aqueous exposure. It was used to identify amoebicides that could potentially be used in a bath to treat fish with AGD.

Paramoeba sp. 'AGD' was suspended in sterile sea-water (negative control) or sterile sea-water containing the chemical. Samples were taken at the commencement of the trial from the untreated negative control suspensions, and then after four hours from the negative controls and chemically treated suspensions. The samples were washed to eliminate the chemical or treatment, and the viable number of *Paramoeba*

determined for each sample by the Most Probable Number method (Cochran, 1950; Parnow, 1972). In these studies the MPN was calculated using an MPN computer program (Genestat, Version 3.1, Procedure: Dilution). The amoebicidal activity of the compound was assessed by comparing the viable number of cells present after four hours exposure to no chemical, with the number present in the chemically treated cell suspension. All tests and controls were performed in duplicate. For a detailed methodology see Method 2.9.3.

A standard two sample t-test was performed to determine if the mean of the control was significantly different from that of the exposed sample. Statistical analysis was undertaken by F. Choo, Statistical Consulting Centre, Melbourne University. The results were also expressed as a percentage reduction, determined by calculating the difference between the mean viable *Paramoeba* number after exposure compared to the negative control at the same sampling time.

Unless otherwise stated, chemicals were tested at 30µg/ml¹ and an exposure time of four hours². Chloramine-T was tested at 66 ppm, as suggested in the literature (Herwig, 1979). Zephiran was tested at an upper limit of 30 ppm (D. Cameron, Pers. Comm.). Other variations in treatment time and concentration are described below. No more than five tests (including the control) were processed a one time, to reduce the errors associated with handling numerous dilutions and replicates.

Hydrogen peroxide was tested at concentrations ranging from 1500 ppm to 50 ppm to determine the minimum inhibitory concentration, the upper level was chosen based on reports using the chemical as a delousing agent in Atlantic salmon (Bruno, 1992; Thomassen, 1992). The minimum exposure time was determined by stepwise reductions in exposure time. The response of hydrogen peroxide to increased *Paramoeba* dose was also tested by increasing the initial number of *Paramoeba* from 15,000 to 60,000 per ml, and increasing the dilution series accordingly.

¹ This upper concentration was determined by consultation with industry and research veterinarians.

² The maximum time fish in the field can safely be contained in a liner without additional oxygen (D. Cameron, Pers. Comm.).

The amoebicidal effects of ozone were tested by bubbling ozone through a suspension of *Paramoeba* for 2-4 hours. Ozone was generated by UV irradiation of air, using the ozone generating system described by Lewis (1993). Ozone levels were determined by a commercial detection kit (Lovibond, U.K.). The number of viable *Paramoeba* was determined before treatment and after 4 hours exposure.

To determine the amoebicidal effect of sea-water at pH 6 the pH of sea-water was lowered to pH 6 using both hydrochloric acid and acetic acid, and the number of viable *Paramoeba* calculated after four hours exposure. This pH level was chosen as the lowest pH that could be tolerated by fish (B. Munday, Pers. Comm.).

To ascertain whether freshwater was amoebicidal, *Paramoeba* were exposed to filter sterilised R.O. water³ (Appendix 1.2) for two and four hours, and the number of viable *Paramoeba* determined as described.

5.3 RESULTS

5.3.1 Growth inhibition assay

The minimum inhibitory concentration (MIC) of the chemicals that inhibited the growth of *Paramoeba* sp. 'AGD', within the range tested, are shown in Table 5.2. Ethanol was found to be amoebicidal at concentrations of 10,000 ppm and greater, hence chemicals that were solubilised in ethanol were concentrated in 5,000 ppm a level not amoebicidal to *Paramoeba* sp. 'AGD'. Survival of the bacterial substrate, *S. maltophilia*, was observed in each trial to ensure that the chemical did not kill the bacterial substrate inhibiting the *Paramoeba*. *S. maltophilia* did not grow in the presence of Juglone, hence *E. coli* was used as the bacterial substrate in this trial. Some of these chemicals were selected for testing by the contact inhibition assay.

³ R.O. water was used in place of freshwater to ensure that the water was free of contamination that may have influenced the results. In this experiment the term freshwater has been used in the results, discussion and conclusion.

Table 5.2: Chemicals able to inhibit the growth of *Paramoeba* sp. ‘AGD’ in the growth inhibition assay

Chemical	MIC
Mefloquine hydrochloride	≤5µg/ml
Nifurpirinol	≤5µg/ml
5-Chloro-7-iodo-8-hydroxyquinoline	≥5µg/ml
8-hydroxyquinoline	≥5µg/ml
Fumagillin	≥10µg/ml
Levamisole	≥10µg/ml
Quinoline	≥10µg/ml
Narasin	≥15µg/ml
Naphthalophos	≥15µg/ml
Nitrothiazole	≥15µg/ml
Pyrimethamine	≥15µg/ml
Nitroimidazole	≥20µg/ml
Quinine hydrochloride	≥20µg/ml
Quinacrine hydrochloride	≥20µg/ml
Sulphaquinoxaline	≥20µg/ml
Alcohol /Ethanol	≥10,000 ppm

5.3.2 Contact inhibition assay

Initially, the two sample t-test was used to calculate if there was significant difference ($P \leq 0.05$) between the viable number of *Paramoeba* present after exposure to the chemical and viable number present in the control. However, this method of analysis was not appropriate for the data generated in this study, for a number of reasons. Exposure to some chemicals resulted in total loss of viability, which could not be statistically analysed. Duplicate tests often gave the same viable number, therefore the standard deviation and standard error could not be calculated, both values required for the two sample t-test. When able to be calculated the two sample t-test often found no significant differences between the test and control with chemicals that showed large reductions in *Paramoeba* numbers. Tables 5.3 and 5.4 demonstrate the wide confidence limits observed and the application of the two sample t-test to the same data.

Due to the problems associated with statistical analysis it was suggested that the results of chemical exposure be expressed as a percentage reduction compared to the control, the reduction only being considered significant if the confidence limits were separate and did not overlap (F. Choo, Pers. Comm.). Table 5.5 shows the percentage reduction in viable *Paramoeba* sp. ‘AGD’ after four hours exposure to selected chemicals.

Table 5.3: MPN results of a selected chemical trial, demonstrating the wide confidence limits commonly observed

Chemical	MPN	95% Confidence Limits	
		Upper	Lower
Quinacrine	2437	1405	4073
	2657	1540	4417
Pyrimethamine	1297	759.7	2135
	2437	1437	3970
Sulphaquinoxaline	5545	3243	9093
	8376	4717	14386
Control	9665	5409	16743
	11236	6405	19098

Table 5.4: Example of the two sample t-test analysis for same chemical trial

Chemical	Mean	Standard deviation	Standard error (mean)	Significance ('P')
Quinacrine	2547	156	110	0.064
Pyrimethamine	1867	806	570	0.072
Sulphaquinoxaline	6961	2002	1415	0.28
Control	10451	1111	785	NA

Table 5.5: Percentage reduction in viable number of *Paramoeba* sp. ‘AGD’ after four hours exposure in the contact inhibition assay

Chemical	Percentage reduction	Confidence limits	Significant reduction
8-Hydroxyquinoline (30µg/ml)	99.47	separate	Yes
Zephiran (30 ppm)	98.40	separate	Yes
Pyrimethamine (30µg/ml)	82.14	separate	Yes
Quinacrine (30µg/ml)	75.63	separate	Yes
Chloramine-T (66 ppm)	74.70	separate	Yes
5-Chloro-7-iodo-8-hydroxyquinoline (30µg/ml)	72.85	separate	Yes
Alcohol (10,000 ppm)	66.40	separate	Yes
Quinoline (30µg/ml)	48.47	overlapping	No
Sulphaquinoxaline (30µg/ml)	33.30	overlapping	No
Narasin (30µg/ml)	+4.11	overlapping	No

Table 5.6 shows the minimum inhibitory concentration and exposure time of hydrogen peroxide in the contact inhibition assay. The minimum inhibitory concentration of hydrogen peroxide was 100 ppm exposed for 30 minutes. Increasing the number of *Paramoeba* cells from 15,000 to 60,000 in the assay did not appear to reduce the efficacy of the treatment *in vitro*. No significant inhibition was observed at 50 ppm.

Table 5.6: The minimum inhibitory concentration and exposure time of hydrogen peroxide tested by the contact inhibition assay

Hydrogen peroxide Concentration	Time (hours)	% Reduction	Significant
1500 ppm	4	100	Yes
1000 ppm	2	100	Yes
500 ppm	2	100	Yes
250 ppm	2	100	Yes
100 ppm	2	100	Yes
100 ppm (60,000 <i>Paramoeba</i> /ml)	1	100	Yes
100 ppm	30 minutes	100	Yes
50 ppm	1	46.86	No
50 ppm	30 minutes	6.56	No

The amoebicidal effects of freshwater, ozone, and sea-water at pH 6 are shown in Table 5.7. Ozone totally inactivated *Paramoeba* at concentrations between 0.04 - 0.1 ppm. No significant inhibition was observed at ozone concentrations between 0.02-0.08 ppm. Ozone levels were difficult to maintain and standardise with the equipment available to us, therefore was impossible to determine the minimum concentration of ozone lethal to *Paramoeba*.

Lowering the pH with either hydrochloric or acetic acid had little effect on viability. Reduction of the pH of sea-water using concentrated hydrochloric acid was difficult to achieve particularly when a specific pH was required; the stability of the achieved pH was also difficult to maintain as the pH tended to drift towards neutral over a period of time. Hence hydrochloric acid to reduce pH was subsequently abandoned. Lowering the pH with acetic acid was achieved more easily and tended to be more stable over time.

Freshwater totally inactivated *Paramoeba* after 2 hours exposure. This test was repeated and the results found to be reproducible.

Table 5.7: Amoebicidal effects of freshwater, ozone and sea-water at pH 6, tested by the contact inhibition assay

Treatment	Time (hours)	Percentage reduction	Confidence limits	Significant % reduction
Freshwater	4	100	N/A	Yes
Freshwater	2	100	N/A	Yes
Ozone (0.01-0.02 ppm)	4	46.79	overlapping	No
Ozone (0.04-0.1 ppm)	4	100	separate	Yes
Sea-water at pH=6	4	46.50	overlapping	No

5.4 DISCUSSION

The overall aim of this part of the study was to identify potential amoebicidal chemicals for the treatment of AGD and screen them in specially developed *in vitro* assays. Chemicals identified as amoebicidal in this study would then be considered for trials on fish in the field⁴. This is not the conventional pathway of identifying chemical treatments for disease. Neal (1983) suggested that the development of anti-amoebic compounds should be preceded by the study of parasite-specific metabolic pathways and their inhibition, followed by whole parasite *in vitro* studies, experimental *in vivo* models and finally clinical trials. However, this pathway cannot always be followed, due to the time consuming nature and cost of studying parasite-specific metabolic pathways, and the relative urgency to identify a treatment. This is particularly true in AGD, with nothing being found in the literature regarding the metabolic pathways of *Paramoeba* species, and no chemical treatment for AGD being available. The small size of the Tasmanian industry, its economic youth and the unique nature of this disease precluded costly time consuming research.

⁴ The information gained from this study was intended to identify potential chemotheraputents for the treatment of AGD. How these drugs would be administered *in vivo*, at what doses and duration, and whether preliminary toxicity trials were to be undertaken were a matter for Saltas, and the researchers concerned. The term ‘field trial’ has been used to denote any *in vivo* application of the chemicals.

The strategy used in this study was to investigate chemicals identified by past researchers (Percival and Foster, 1988), or used in the therapy of protozoan and parasitic infections in animals and humans. It was hoped to identify chemicals, or a group of class specific chemicals, amoebicidal to *Paramoeba in vitro*. This approach is a recognised method of selecting chemicals for *in vitro* testing, having been employed by Griffin (1989) to select chemicals to control protozoan parasites of fish. A brief description of the chemicals and how they may work has already been given. The chemicals were tested without regard for the pharmacodynamics, as these are not always unequivocally known, or the pharmacokinetics. Griffin (1989) also suggests that the selection of chemicals to treat fish should be inexpensive, water soluble, quick acting, short-lived, harmless to other biota, and leave no residues in the fish. These factors were not considered at the screening stage of this study, although some of these factors were considered when deciding which chemicals would be tested by the more involved contact inhibition assay, and ultimately which chemicals were recommended for field trials.

There are a number of methods used to treat infections in fish. The most popular are the use of medicated feeds and medicated baths. Antibiotics such as oxytetracycline, oxolinic acid and erythromycin are commonly used in medicated feeds to treat bacterial infections of fin fish such as furunculosis, vibriosis and streptococcosis (Carson, 1990). Medicated feeds have also been used to treat systemic infections in fish caused by *Hexamita* and Coccidial species (Langdon, 1990). In general, ectoparasites are treated with medicated baths or dips, the most common of which are formalin, malachite green and copper sulphate (Sedgewick, 1988), although baths using anthelmintic drugs such as praziquantel and febendazole have been used for external metazoan infections of fin fish (Langdon, 1990). Oral or immersion vaccination, administered in a bath treatment, is widespread in the salmon farming industry (Horne, 1997). In Tasmania a locally produced oral vaccine 'Anguillvac-c', made from *V. anguillarum*, has been successfully used to immunise smolt against vibriosis (Munday *et al*, 1992). Injectable vaccination is slow, labour intensive and requires prior anaesthesia of fish, and it was initially thought feasible only for valuable brood stock (Austin and Austin, 1993). However, recently the use of injectable vaccines has become widespread throughout the salmon farming industry

(Horne, 1997), particularly against the bacterium *Aeromonas salmonicida*, the causative agent of furunculosis (Ellis, 1997). Vaccination by injection is inherently expensive; and it is not known if the cost of an injectable vaccine for AGD (if it became available) would be cost efficient compared to the cost of freshwater bathing.

Administering a chemical in the feed would be the easiest method of controlling AGD and comparable in cost to freshwater bathing providing the cost of the drug was less than \$1000/kg (Alexander, 1991). However it is the method with the most inherent problems. The chemical would have to be non-toxic, palatable, absorbed from the gut, and be able to penetrate the gills and mucus in amoebicidal or inhibitory levels. The residence times of the chemical in the fish would need to be low. Stability in feeds, cost, environmental and personal toxicity, and residence times in the fish are all important factors when considering a medicated feed treatment.

Medicated baths involve bathing fish for a short period of time in salt-water containing the treatment or chemical. This method would involve fitting a liner around the pen and adding the amoebicide directly to the water. Such a procedure would be advantageous, since it would reduce labour costs and reduce handling the fish, thereby minimising stress and skin damage that can lead to secondary opportunistic bacterial infections. Effective treatments would need to be amoebicidal over a short exposure period and be able to penetrate the mucus associated with the gills. The therapeutic effects of freshwater baths are not yet fully understood, however any amoebicidal chemicals identified would need to emulate the therapeutic effects of freshwater bathing. In addition, chemicals would need to be soluble in sea-water, non-toxic to the fish and the environment, and remain active in sea-water. As with all treatments, cost and availability of the chemical would also be limiting factors. Griffin (1989) suggested that purity of the chemical should also be considered as this can vary considerably between batches and suppliers.

To investigate the amoebicidal effects of chemicals selected, a two-staged approach was taken. Most of the chemicals were tested for their amoebicidal activity to *Paramoeba* over a 14 day period using the growth inhibition assay. This method was able to quantify the effects of the amoebicidal activity giving a minimum inhibitory concentration of the chemical within the range tested. The highest level of chemical tested was 30µg/ml, selected as the maximum concentration of chemical that may be achievable in tissues or serum (B. Munday, Pers Comm.) or could be used economically in a bath treatment. Screening assays of this type do not give an indication of the rate of inactivation, or the proportion of cells affected per unit time. It was thought that the data from these trials would give some indication of the efficacy of the chemical as a medicated feed in fish, and thus was used to recommend chemicals for field trials.

Selected chemicals found to be amoebicidal in the growth inhibition assay were further investigated using the contact inhibition assay to predict their likely success as a bath treatment. Some chemicals found amoebicidal by the growth inhibition assay were not tested by the contact inhibition assay, the reasons for this will be discussed further in this section. Chemicals more suited to exogenous administration, such as hydrogen peroxide, chloramine-T, Zephiran and ozone, were investigated for amoebicidal activity only by the contact inhibition assay. Chemicals were tested either at 30µg/ml or at a level suggested for use in the literature. The exposure time of the chemicals to *Paramoeba* was set at a maximum of four hours, the maximum time the fish in the field can safely be contained in a liner without additional oxygen (D. Cameron, Pers. Comm.). This method relied on comparing the viable number of *Paramoeba* exposed to the chemical with the number viable without treatment. Failure to grow after exposure for 4 hours was considered a selection criterion for a chemical that may have a lethal effect in a bath treatment of fish.

5.4.1 Growth inhibition assay

The growth inhibition assay developed in this study is a reproducible and rapid method, allowing mass screening of chemicals, for potential amoebicidal activity to *Paramoeba* species. The continued exposure to chemicals employed by this method was thought to closely mimic the process of in-feed medication, whereby the *Paramoeba* would be exposed to the drug in the gills for long periods.

Incorporation of the chemicals into the solid media is commonly used in bacteriology as a selection media, to differentiate strains and species of bacteria. Similar methods have been employed to determine the amoebicidal activity of chemicals against *Entamoeba histolytica*. Youssef (1964) tested chemicals by placing filter discs soaked in the chemicals onto actively growing cultures of *E. histolytica*, in the presence of a bacterial growth substrate, and observing for zones of inhibition. Myjak (1972) developed a similar technique where the zones of inhibition were measured and compared.

However, there were a number of important limitations with this method. The obligatory presence of the bacterium *S. maltophilia* in this method introduced the problem of potential antibacterial activity of the drugs employed. Anti-bacterial activity was rarely encountered using this method, and was observed by the absence of bacterial growth on the chemical incorporated agar plates. Juglone was the only drug that exhibited anti-bacterial activity, and for this chemical *E. coli* was substituted as the bacterial growth substrate. However it was not known if the presence of the bacterial substrate, or other components in the media such as malt or yeast, negatively affected the amoebicide assay by reducing the toxicity of the test chemical. The effect of pH on the activity of the chemicals was not determined. Neal (1983) suggested that pH should be monitored as it is known that emetine is more active in an alkaline medium than an acidic media. However, the effects of pH on the individual chemicals were most often not reported in the literature, hence pH monitoring was not considered necessary. Another important limitation was that screening assays of this type do not give an indication of the rate of inhibition or inactivation, or the proportion of cells affected per unit time.

Of the 37 chemicals tested by the growth inhibition assay, 15 were identified as inhibitory to *Paramoeba*. In addition, ethanol at concentrations $\geq 10,000$ ppm, was found to be inhibitory, hence the concentration of alcohol as a solvent for selected chemicals needed to be reduced to allow the activity of the chemicals to be determined. Ethanol was used as a solvent for only four of the 15 inhibitory chemicals, and it is not known if the presence of ethanol positively contributed to the inhibitory effect. While a number of the chemicals screened were not inhibitory at the concentrations tested, they may be amoebicidal or amoebistatic at higher concentrations.

The only class specific chemicals identified in this study as inhibitory to *Paramoeba* belonged to the quinoline group of compounds (8-hydroxyquinoline, 5-chloro-7-iodo-8-hydroxyquinoline and quinoline). All three members of this group showed similar high amoebicidal activity by this testing method. Mefloquine hydrochloride, from the quinoline-methanol class of drugs also showed high amoebicidal activity. However, the aminoquinolines, of which chloroquine and primaquine belong, had no inhibitory effect on *Paramoeba*. Monensin showed no inhibitory effect, however its chemical relative Narasin was inhibitory. This was also true of nitrofurantoin which was not amoebicidal at the concentrations tested compared to the inhibitory activity of the other nitrofurans tested, nifurpirinol and nitrothiazole. Similarly 4-nitroimidazole was inhibitory but other nitroimidazoles such as carnidazole, metronidazole and tinidazole showed no inhibition at the concentrations tested. Further *in vitro* investigation of other chemicals belonging to these groups may identify additional chemicals amoebicidal to *Paramoeba*.

Although a range of inhibitory chemicals was identified in this study, there does not appear to be a common cellular process that is targeted by the drugs. The precise mode of action of many of the chemicals tested was either unknown, or postulated. However three of the 15 inhibitory chemicals, 4-nitroimidazole, quinine hydrochloride and quinacrine hydrochloride, are thought to intercalate with the DNA, inhibiting DNA and/or RNA synthesis. The following is a list of the modes of action for each of the amoebicidal chemicals found inhibitory: the 8-hydroxyquinolines act

by chelating ferrous ions; quinoline acts by disrupting electron transport in the mitochondrial cytochrome system; levamisole paralyses the nervous system of nematodes; pyrimethamine blocks the action of the enzyme dihydrofolate reductase; narasin is active by rendering membranes permeable to sodium and potassium ions; the action of sulphaquinoxaline is thought to be due to competitive inhibition with p-amino benzoic acid; the modes of action of nifurpirinol, mefloquine hydrochloride, naphthalophos, and nitrothiazole are not known. The chemicals identified as inhibitory to *Paramoeba in vitro* have been used to treat a diversity of parasites, some unicellular and others multicellular. Published data indicates that these compounds affect different cellular processes but it is possible that there is some commonality with regard to *Paramoeba*, since they may all exert an antagonistic effect on a single, common cellular process, as yet unidentified.

The aim of the growth inhibition assay was to identify chemicals that could be used in feed to treat fish infected with amoebic gill disease and to select which chemicals were to be tested by the contact inhibition assay. Chemicals found amoebicidal in this assay were selected or eliminated from further *in vitro* testing, or recommended for field based trials, based on a number of factors; past use and success in fish species in treating diseases caused by protozoa, ability to be absorbed from the gut, and toxicity. Cost of the chemical was also considered.

In this study, mefloquine hydrochloride ('Lariam') showed strong amoebicidal activity *in vitro*, being able to inhibit growth of *Paramoeba* at $\leq 5\mu\text{g/ml}$. The chemical does not appear to be commercially available in its pure form, hence in this study 'Lariam' tablets were crushed and solubilised to yield the component chemical. It is not known if carrier chemicals present in the tablets were the cause of the amoebicidal activity observed. Commercial availability may be a problem, and the cost may be prohibitive. Mefloquine has not been used to treat fish species in the past, however based on the high amoebicidal activity to *Paramoeba* and possible ability to be absorbed from the gut, its investigation as a feed medication was highly recommended. This chemical was not considered for testing by the contact inhibition assay as the cost of the chemical may preclude its use as a bath medication, and its current use as a chemotherapeutant to treat malaria.

Nifurpirinol has been used primarily as an external treatment for fin rot, bacterial gill disease and external protozoan parasites (Herwig, 1979). The drug has also been given orally to treat bacterial diseases in salmonids caused by *Aeromonas* and *Vibrio* species, and hexamitiasis in Siamese fighting fish (Ferguson and Moccia, 1980; Sedgewick, 1988). Nifurpirinol has been identified as a carcinogen (Kimura *et al*, 1989) and is no longer considered a safe chemical for the treatment of fish diseases. It was not recommended for further laboratory or field based trials, despite its high amoebicidal activity in this study. Another nitrofur, nitrothiazole was identified as amoebicidal to *Paramoeba* in this study, although its activity was less than Nifurpirinol. Very little is known about nitrothiazole, although absorption is thought to be good. It is a relatively inexpensive chemical and thus was recommended for field testing as a feed medication. The success of nifurpirinol as a bath medication in fish suggests that this chemical may also be similarly effective, and thus the inhibitory effects of this chemical were further investigated by the contact inhibition assay. The results of this study suggest that the *in vitro* investigation of other nitrofurans, such as furazolidone and nifurtimox, might be justified (see Looker *et al*, 1987).

Both the hydroxyquinolines and quinoline showed similar high levels of amoebicidal activity in this study. None of these chemicals have been used in the treatment of fish. As the hydroxyquinolines are poorly absorbed from the gut, it was expected that the concentration in the gills might be too low to be amoebicidal. The hydroxyquinolines tested in this study were selected for further testing by the contact inhibition assay to predict their efficacy in a chemical bath treatment; and were not recommended for medicated feed trials. Quinoline is a highly toxic chemical to humans and was not recommended for field based trials. However, further testing by the contact inhibition assay was performed to compare the results of exposure to this chemical and the hydroxyquinolines. Clamoxiquin is a member of the 8-hydroxyquinoline group that has been used as an enteric amoebicide in man. It has been found to produce significant reductions in whirling disease pathology (caused by the protozoan *Myxosoma cerebralis*) in rainbow trout (Alderman, 1986). During the present study it proved to be unobtainable. The investigation of Clamoxiquin and other chemicals in the quinoline group appears to be warranted.

Levamisole has been successful as a medicated bath in the treatment of the skin parasitising monogenean, *Gyrodactylus* sp. in sticklebacks (Schmahl and Taraschewski, 1987), but has been ineffective in treating gyrodactylosis as an oral treatment in rainbow trout (Tojo and Santamarina, 1998a). It has also been tested as an oral treatment in rainbow trout against infection with the gill and skin parasite *Ichthyobodo necator* and the intestinal parasite *Hexamita salmonis*, but with no effect (Tojo and Santamarina, 1998b and c). Levamisole has been shown to act as an immunostimulant and is currently being used in adjuvant therapy to stimulate the immune system of cancer patients (Penna and Nordlinger, 1996; Saltz and Kelsen, 1997). Its use as an immunostimulant in fish has been tested and shown to increase the resistance of fish to a number of bacterial pathogens (Sakai, 1999). Levamisole was highly recommended for testing as an in-feed medication in the field due to the amoebicidal activity of levamisole observed in this study, combined with its ability to be absorbed from the gut and to stimulate the immune system. At the time of this study further investigation by the contact inhibition assay was not considered warranted. However in hindsight the amoebicidal activity of short term exposure to levamisole may have identified this chemical as a potential bath medication.

Oral treatment with fumagillin is effective for controlling various microsporidian and myxosporean infections in fish, but some toxicity problems have been reported (Molnár *et al*, 1987; Wishovsky *et al*, 1990; Yokoyama *et al*, 1990; Kent and Dawe, 1994; Higgins and Kent, 1996; le Gouvello *et al*, 1999; Speare *et al*, 1999a). A synthetic analogue of fumagillin has also been shown to be effective in controlling two microsporidian pathogens of salmon (Higgins and Kent, 1998). As fumagillin is readily absorbed from the gut and shows high amoebicidal activity to *Paramoeba* it was recommended for trials as an in-feed medication in the field. It was not considered for testing by the contact inhibition assay at this stage.

Pyrimethamine has been tested as an antiprotozoal agent against the skin parasites *Cryptocaryon irritans* and *Ichthyophthirius* species in fish with little success (Herwig, 1979). Pyrimethamine combined with sulphaquinoxaline has been shown to be effective *in vitro* against a ciliated protozoan (*Anophryoides haemophila*) that causes

disease in lobsters, causing a reduction in ciliate motility and inducing cell lysis of the parasite (Novotny *et al*, 1996). The same combination has been tested as an oral treatment against the microsporidian gill pathogen *Loma salmonae* in rainbow trout with no success (Speare *et al*, 1999a). Sulphaquinoxaline has been used as a bath treatment to treat rainbow trout infected with *I. necator* but was unsuccessful (Tojo *et al*, 1994b). As pyrimethamine and sulphaquinoxaline are readily absorbed from the gut, both were recommended for field trials as an in-feed medication, although sulphaquinoxaline was given a low priority. They were also selected for further testing by the contact inhibition assay. However like mefloquine hydrochloride, the cost of pyrimethamine could be prohibitive, hence its use in combination therapy with the less expensive sulphaquinoxaline should also be considered.

There are no reports of narasin being used to treat fish infections. However it has been found to be inhibitory *in vitro* to an *Enterococcus*-like species pathogenic for rainbow trout in Australia (Carson and Statham, 1993). Narasin was recommended for field trials as an in-feed medication due to industry interest in this chemical, despite reports that it is poorly absorbed from the gut. Narasin was selected for further testing by the contact inhibition assay.

Naphthalophos is an organophosphate and, as the use of organophosphates in the marine environment is not recommended, this chemical was eliminated as a candidate for field testing (D. Cameron, Pers. Comm.).

The use of quinine hydrochloride in medicated feed has been tested against *Loma salmonae* (gill parasite) infection in rainbow trout with promising results (Speare *et al*, 1998). Medicated feed containing quinine has also been found to kill the skin inhabiting trophozoite stage of *Ichthyophthirus multifiliis* in ornamental fish (Schmahl *et al*, 1996). Quinine hydrochloride is readily absorbed from the gut and although the amoebicidal activity observed in this study was lower than observed for other chemicals, it was selected as a low priority for field testing as a medicated feed and was not investigated using the contact inhibition assay.

Quinacrine hydrochloride has been used to treat a variety of skin parasites, principally the protozoa *Cryptocaryon irritans*, *Henneguaya*, *Ichthyophthirius*, *Oodinium ocellatum* and a number of sporozoan species (Herwig, 1979). It has also been used as a bath treatment to treat rainbow trout infected with the flagellate protozoan *I. necator* but was unsuccessful (Tojo *et al*, 1994b). Quinacrine is readily absorbed from the gut. Further investigation of this chemical in the field as a medicated feed treatment was recommended, again as a low priority.

Of the four nitroimidazoles tested only 4-nitroimidazole was found to be amoebicidal to *Paramoeba* at the concentrations tested. Oral treatment with metronidazole, a nitroimidazole, has been shown to completely eradicate *I. necator* and *H. salmonis* infections in rainbow trout (Tojo and Santamarina, 1998 b and c). It has also been evaluated in the treatment of *L. salmonae* and *Gyrodactylus* species infections in rainbow trout but with little success (Tojo and Santamarina, 1998a; Speare *et al*, 1999a). None of the other nitroimidazoles have been tested in fish species. 4-Nitroimidazole was recommended as a low priority for testing in the field as a feed medication. Further *in vitro* evaluation of other nitroimidazoles, such as dimetridazole, secnidazole, benzimidazole and ronidazole is warranted (see Tojo and Santamarina, 1998 b and c).

To date a number of the chemicals recommended in this study have been tested in the field as medicated feeds. Alexander (1991), tested a number of the chemicals selected by this study in field based trials, prior to these results becoming available. Of the amoebicidal chemicals identified only fumagillin and quinacrine were tested. Fumagillin was tested at a dose rate of 0.1% in the feed for three weeks, and the dose then increased to 0.3% for one week, with no discernable effect on the development of AGD. Fumagillin was found to be toxic to the fish, causing high mortalities during and post medication, and histological examination showed skin thinning and subsequent bacterial infection, plus depletion of the haemopoietic tissue in the kidney. However, it should be noted that the treatment with fumagillin was commenced when the fish were already infected with a moderate level of AGD, which may have compromised the results. The toxic effects of fumagillin within the range tested by

Alexander have been previously reported (Wishovsky *et al*, 1990). However, there have been a number of reports of successful treatment of various myxosporean and microsporean pathogens of salmonids (Kent and Dawe, 1994; Higgins and Kent, 1996; le Gouvello *et al*, 1999; Speare *et al*, 1999). A synthetic analogue of fumagillin (TNP-470) has also been shown to be effective in controlling two microsporidian pathogens of salmon (Higgins *et al*, 1998). The success of these treatments, without the associated mortalities observed by Alexander (1991), appears to be due to the use of much lower fumagillin dose rates, and a reduction in the duration of the treatments. Daily doses of about 3-10 mg/kg fish are recommended for salmonids (Higgins and Kent, 1996). It is clear from the above publications that careful consideration of the dose, duration, and level of initial infection in fish should be considered when using fumagillin to treat salmonids. Hence, further investigation of fumagillin, and the synthetic analogue TNP-470, is required before it can be ruled out as a potential treatment for AGD in the field. Quinacrine was tested as both a medicated feed and medicated bath treatment, whilst fumagillin was only tested as a feed medication. Quinacrine hydrochloride as a medicated feed was found to have limited efficacy in the early stages of AGD, the author suggesting that higher doses than those administered might be effective. As a bath treatment quinacrine was not effective in controlling AGD. However, the use of quinacrine may not be desirable as it was found to be highly absorbed, accumulating in the tissue of the fish and turning it a bright yellow.

Cameron (1992) tested narasin, juglone, 8-hydroxyquinoline and levamisole as feed medications, based on their amoebicidal activity to a *Platyamoeba* species isolated from the gills of fish with AGD and initially thought to be the causative agent of AGD (see Howard and Carson, 1991). Only with narasin was the level of AGD reduced, but not completely eliminated, however some palatability problems were encountered. Cameron recommended further investigation of this chemical as a feed medication. It is worth noting however, that some problems with the level of infection and timing of the treatments were encountered during the field trials conducted by both Cameron and Alexander (1991).

Recently, levamisole added to freshwater baths was associated with a significant decrease in the mortality of fish exposed to AGD, but no effect was observed with oral supplementation with levamisole (Zilberg *et al*, 2000). These trials were conducted in tanks, and fish infected by cohabitation using methods similar to the ones already described (see Chapter 4). The authors suggested that this response was due to enhancement of the non-specific immune system. Field trials of levamisole added to freshwater baths failed to offer any increased protection to AGD (Clark and Nowak, 1999).

It is important to remember that the key to the success of these chemicals as feed treatments relies heavily on their ability to reach amoebicidal levels in the gills of the fish. Further investigation of the relationship of the concentration required in the feed to reach amoebicidal levels in the gills may need to be performed. Timing of medication should also be investigated as medicated feeds are unlikely to be therapeutic if infection is too advanced as the levels of mucus on the gills may negate the amoebicidal effects of the drug. To date, most trials of feed medications have been performed in the field on pens of fish naturally exposed to *Paramoeba*. The development of a method of infection using cohabitation of AGD infected fish with naïve fish now allows tank trials to be undertaken where the rate of infection can be controlled and the effects of the medications more closely observed. Tank trials would also be less labour intensive to conduct and hence less costly, allowing more parameters to be tested.

5.4.2 Contact inhibition assay

The contact inhibition assay was developed to determine the amoebicidal activity of selected chemicals in a short-term bath exposure. This method was thought to closely mimic the activity of these chemicals if used in a medicated bath treatment in the field. This type of assay was able to provide an indication of the rate of inhibition or inactivation, and the proportion of cells affected per unit time.

Viable counts were determined using the Most Probable Number (MPN) method, a method used extensively to estimate bacterial numbers in water (Madden and Gilmour, 1995; Schaub and Vangemerden, 1996; Teske *et al*, 1996). The MPN method has also been used to determine the number of pathogenic amoebae in freshwater (Robinson *et al*, 1990) and the number of Gymnamoebae in marine sediments (Butler and Rogerson, 1995). The accuracy of this method relies on two assumptions, that organisms are randomly distributed in the suspension and that if the organism is incubated in the appropriate medium the sample will always show growth if one or more organisms are present (Cochran, 1950). The accuracy of the count depends on three basic factors: the dilution series must be sufficiently wide for one or more dilutions to give some positive and some negative results; the smaller the dilution factor the greater the precision; increasing the number of replicates narrows the confidence limits and increases the precision of the count (Parnow, 1972; Best, 1990; Beliaeff and Mary, 1993).

In this study all efforts were made to select the best combination of parameters to produce an accurate count of *Paramoeba*. In addition, improvements made in the culture of *Paramoeba* (see Chapter 3) have increased the likelihood that any sample containing *Paramoeba* would show growth. The dilution factor and number of replicates used to determine the MPN in this study were much larger than those employed by other researchers in the enumeration of amoebae from soil and sediments (Robinson *et al*, 1990; Butler and Rogerson, 1995). However, practical testing revealed a number of inherent problems with this methodology. Although the results were repeatable between duplicates and experiments, the confidence limits were quite wide. The confidence limits could have been narrowed if significantly more replicates were employed, however this would have made the experiments far too large and costly. In addition, any increase in the number of replicates may have introduced greater experimental error due to the sheer size of the experiments.

Statistical analysis of the results of the MPN proved difficult. Although results were expressed as percentage reduction, it was a difficult task to prove what level of reduction was significant. Examination of the literature did not elucidate a suitable method of statistical analysis, with most researchers accepting the numbers given by this method. In this study results were only considered significant if the confidence limits did not overlap, examination of the corresponding reductions demonstrated that reductions less than approximately 50% could not be considered significant, as the confidence limits overlapped at this percentage reduction. This method of analysis was felt to be accurate, as chemicals with less than 50% reduction would be unlikely to produce the desired amoebicidal activity in the field.

The results of freshwater testing established *in vitro* that freshwater is lethal to *Paramoeba* within 2 hours. Prior to this experiment the lethal effects of freshwater had been postulated, but not confirmed. Farms routinely bath AGD infected salmon in freshwater for between 2-4 hours (J. Smith, Pers. Comm.). Cameron (1993) reported that the maximum salinity for effective bathing was 4 ppt for baths of 2.5 hours duration. These observations confirm that one of the therapeutic effects of freshwater is its lethal effect on *Paramoeba*. It is unlikely however that the therapeutic effect of freshwater rests solely with its lethal effect on *Paramoeba*, as mucus is also removed from the gills during freshwater bathing, restoring osmotic balance to affected fish (Jones, 1988; Munday *et al*, 1990).

Of the 12 chemicals or treatments tested, eight were found to be significantly amoebicidal to *Paramoeba* when exposed for four hours. Seven of the 12 chemicals were selected due to their amoebicidal effects in the growth inhibition assay and of these, four were significantly amoebicidal in this assay, and two showed some reduction in *Paramoeba* numbers which were not considered significant. With the exception of narasin, these results validate those found by the growth inhibition assay. Narasin was not amoebicidal within the four hours, which suggest that a much longer exposure time may be required for narasin to be amoebicidal.

Hydrogen peroxide was identified as the most promising alternative treatment identified by this study. The results demonstrated that hydrogen peroxide totally inactivated *Paramoeba* at 100 ppm and an exposure time of 30 minutes. Hydrogen peroxide is a recognised bacteriostat and effective sporicide (Baldry, 1983). Its use to treat ectoparasites on freshwater fish was reported as early as 1920 (Thomassen, 1992). More recently, the use of hydrogen peroxide as a delousing treatment for sea-lice infected Atlantic salmon has been described (Thomassen, 1992; Bruno, 1992a). Thomassen (1992) used concentrations of 1500 ppm hydrogen peroxide for 20 minutes on Atlantic salmon in sea-water and reported a removal rate of sea-lice of 85-100%. Since this study hydrogen peroxide has been found effective in treating rainbow trout experimentally infected with *Flavobacterium branchiophilum*, the causative agent of bacterial gill disease (Lumsden *et al*, 1998), in the treatment of channel catfish infected with the fungal disease, saprolegniasis (Howe *et al*, 1999), and in the treatment of rainbow trout experimentally infected with *Flexibacter columnaris* (Speare and Arsenault, 1997). The levels of hydrogen peroxide used varied significantly but were well below the levels reported by Thomassen (1992). Toxicity problems causing increased mortality, gill damage and reduction in the growth rate of treated fish have been reported associated with some treatment levels (Lumsden *et al*, 1998; Derkson *et al*, 1999; Gaikowski, *et al*, 1999; Speare and Arsenault, 1999; Speare *et al*, 1999b).

The mode of action of hydrogen peroxide is not clearly understood. The bactericidal effect is thought to occur as a result of unspecified multiple cellular injuries (Baldry, 1983). Oxygen liberated from hydrogen peroxide as a result of catalase activity has been suggested as the cause of death for protozoa and monogenea (Thomassen, 1992). The action in sea-lice is thought to be due to large amounts of oxygen liberated inside the lice, forming gas bubbles in the gut and haemolymph which causes the lice to detach from the host and rise to the surface of the water, the results of which are usually fatal to the lice (Thomassen, 1992). Hydrogen peroxide is toxic to Atlantic salmon and this toxicity increases with concentration, exposure time and temperature (Thomassen, 1992). Although toxicity can occur, Atlantic salmon can survive exposures of 1500 ppm at 18°C for up to 30 minutes (Thomassen, 1992). Most of this work has been done on large fish and its use on smaller fish and smolt was unknown.

Field testing of hydrogen peroxide was commenced by another researcher soon after the results of this study were made available to industry. Hydrogen peroxide was found to be relatively stable in sea-water at solutions greater than 200 ppm, even at a concentration of 100 ppm reduction was minimal (Cameron, 1994a). Breakdown of hydrogen peroxide was greatest in the presence of the fish and netting. Toxicity testing showed the degree of toxicity increased with increasing temperature, concentration and exposure time, and that larger smolt were more tolerant than small smolt (Cameron, 1994b). Field testing was disappointing with concentrations of 100, 200 and 300 ppm in sea-water failing to control developing AGD. More importantly hydrogen peroxide was shown to have a narrow margin of safety due to the temperature dependant nature of its toxicity to Atlantic salmon (Cameron, 1994c). These findings suggested to Cameron that hydrogen peroxide had little potential as a routine therapy to control AGD in Tasmania.

Ozone has been reported as an effective bactericide and antiprotozoal agent, and its use as a disinfectant for freshwater aquaculture systems has been recognised (Korich *et al*, 1990; Sugita *et al*, 1992; Williams *et al*, 1982). In this study ozone was identified as amoebicidal to *Paramoeba in vitro*, when exposed to concentrations between 0.04 and 0.1 ppm for four hours.

Ozone is a biocide with a short lifespan, breaking down to oxygen and an oxygen free radical, which has biocidal properties due to its high oxidising potential. Salmon are able to tolerate biocidal concentrations of ozone with no apparent harm (Lewis, 1993). Lewis (1993) tested ozone to control the growth of biofouling organisms on salmon nets, a reduction in biofouling only being observed on the net panel immediately above the diffuser, probably due to the enormous dilution effects. Further study was abandoned. These findings suggest that ozone is unlikely to be efficacious as a treatment in the field, due in most part to the inability to produce enough ozone in the pens to be biocidal.

Abrupt changes to the pH of sea-water have also been suggested as possible means of stripping mucus from the gills of infected fish and inactivating amoebae. In this study sea-water, lowered to pH 6, had very little effect on the survival of *Paramoeba*. Sea-water was very difficult to lower from pH 8 to pH 6 and it is likely that this will also occur in the field. This form of treatment showed no potential as a treatment for AGD.

Of the other chemicals found to be amoebicidal, 8-hydroxyquinoline and chloro-iodo-hydroxyquinoline showed enough inhibition to warrant field trials. In contrast quinoline, a chemical relative to the hydroxyquinolines was not significantly amoebicidal, and is not recommended for field trials for reasons already explained. Zephiran (benzylkonium chloride) was not recommended for use due to reported toxicity problems and the unsuccessful use of this chemical in previous investigations (see Chapter 4). Published reports suggested against the use of Zephiran to treat gill diseases, due to the severe gill damage it induces, even at very low concentrations (Hoskins and Dalziel, 1984; Byrne *et al*, 1989). Pyrimethamine also appeared to be worth testing in the field, but the cost of the chemical may be prohibitive. Despite showing significant amoebicidal activity, quinacrine was not recommended for field testing as a medicated bath treatment for reasons already discussed.

Chloramine-T (6 ppm) has been used unsuccessfully to treat AGD in the past (Munday *et al*, 1993). Field trials of chloramine-T are warranted as the amoebicidal concentration is likely to be higher than 6 ppm. Toxicity trials should be undertaken before field trials in AGD fish are attempted, as the level found amoebicidal in this study is likely to be toxic to the fish. Recently chloramine-T was reported to cause extensive gill damage in rainbow trout exposed to 10 and 20 µg/ml twice weekly for one hour each in freshwater (Powell *et al*, 1995). A reduction of the growth rate of rainbow trout after exposure to chloramine-T in fresh water has also been reported (Powell *et al*, 1994). The toxicity of chloramine-T on Atlantic salmon in sea-water, and the effect on growth, may eliminate this chemical before field trials are undertaken.

The poor results observed in field testing of some chemicals, specifically hydrogen peroxide, only serves to highlight the problems that can be encountered when extrapolating *in vitro* activity into field efficacy. A factor that should be considered when testing these chemicals in bath treatments is the increased level of organic matter present in and on the nets containing the fish, as these may interfere with or reduce the activity of, the chemicals being tested. The three stage approach employed by Cameron (1994 a, b, and c) involving testing the stability of the chemical in sea-water, followed by toxicity testing, and finally testing in AGD infected fish, is an excellent approach. The development of a method of infection using cohabitation of naïve fish with AGD infected fish allows tanks trials to be undertaken, eliminating treatments that do not work before costly field trials are undertaken.

5.5 CONCLUSIONS

The growth inhibition and contact inhibition assays developed in this study are effective methods that can be used to determine the amoebicidal activity of chemicals to *Paramoeba in vitro*. Using these methods a number of chemicals were found to be amoebicidal to *Paramoeba in vitro* and were recommended for testing *in vivo* as medicated feed or bath treatments, either in the field, or in tanks using cohabitation to induce AGD (see Chapter 4). Mefloquine hydrochloride, pyrimethamine, nitrothiazole, levamisole, and fumagillin were highly recommended for testing as medicated feed treatments. Quinine hydrochloride, quinacrine hydrochloride sulphaquinoxline, narasin and 4-nitroimidazole were recommended as low priorities for testing as medicated feed treatments. Levamisole, quinacrine hydrochloride and fumagillin have been tested as feed medications by other researchers (Alexander, 1991; Cameron, 1992), the results of which eliminated them in the treatment of AGD. However, fumagillin warrants further investigation as recent reports show its efficacy at much lower dose rates and duration of treatment, without the associated toxic effects and mortalities observed in the field trial undertaken by Alexander (1991). Narasin has also been tested in the field as a feed medication and found to reduce but not completely eliminate AGD (Cameron, 1992). The two hydroxyquinolines and pyrimethamine were recommended for testing as medicated bath treatments; and further investigation of chloramine-T in the treatment of AGD was also suggested.

This study confirmed the lethal effect of freshwater, but it is unlikely that the therapeutic effect of freshwater rests solely with its lethal effect on *Paramoeba*, as mucus is also removed from the gills during freshwater bathing. The amoebicidal chemicals recommended are unlikely to emulate the therapeutic effects of freshwater bathing. Feed and bath medications may only be effective when used prophylactically to prevent or control early stages of AGD, when mucous patches are absent or very few in number. As AGD is diagnosed in the farms by the presence of mucous patches, the development of more sensitive methods of diagnosis may assist in the timing of any chemical treatments employed to control AGD.

To date very few of these chemicals have been tested in field trials as in-feed or bath medications. This is largely due to the disappointing results of the field trials conducted to date, particularly hydrogen peroxide, and a shift in attitude within the Tasmanian industry to the use of chemicals to control disease. Increased competition from larger salmon growing countries means that the Tasmanian salmonid industry must continue to limit its use of chemicals to maintain its niche markets established on the basis of its relatively chemical-free status. As freshwater bathing would continue to be the method of choice to control AGD in Tasmania, the objectives of this next part of the study were to develop a sensitive diagnostic assay, allowing early prediction of AGD in fish; and to investigate any other means of predicting outbreaks or improving the diagnosis of AGD.

It is now unlikely that these chemicals will be investigated further in Tasmania. However, the results may be particularly relevant to other countries where AGD occurs, such as in Ireland, where freshwater bathing is not practical due to the location of the sea-farming sites (see Rodger and McArdle, 1996 and Palmer *et al*, 1997). Further *in vivo* and field investigation of the amoebicidal chemicals identified in this study in Tasmania is warranted, as they may be of specific value occasionally, when freshwater is not available, thus extending the range of treatments for AGD.

Chapter 6. Non-chemical control of AGD

6.1 INTRODUCTION

In the absence of a chemical treatment for AGD the Tasmanian salmonid industry relies on the use of freshwater bathing to control AGD (Munday *et al*, 1990). The first freshwater bath usually occurs 6-10 weeks after the fish are placed into the sea, often when there are no signs of disease (J. Smith, Pers. Comm.). After the first bath, the bathing schedule is determined by gross gill checks of a small number of fish from each pen, and estimating the extent of AGD by the size and number of mucous patches present on the gills (Alexander, 1991). Bathing begins with cages showing the greatest signs of disease and proceeds to all cages; this process is repeated for most of the summer and early autumn months. Freshwater baths are often repeated every 4-6 weeks during the summer months (Clark and Nowak, 1999).

On the farms the process of avoiding outbreaks of severe AGD, and hence the devastating losses that could ensue, requires constant monitoring and bathing of individual fish cages, which is both costly and time consuming. Reliance on gross signs of disease to determine the bathing schedule has inherent risks as these fish can rapidly develop severe AGD.

Definitive diagnosis of AGD is achieved by examining chemically fixed sections of gill tissue provided by the farms and observing them for gill damage and amoebae, consistent with AGD (Roubal *et al*, 1989; Munday *et al*, 1990). Although an accurate assessment of gill health, it has a number of drawbacks: it requires that the fish are sacrificed which is costly in terms of large fish; takes a minimum of one week for the results to be processed and examined; and is costly as sections need to be examined by a trained veterinary pathologist. Fixed gill sections may not always provide an accurate assessment of AGD as AGD-like changes in the gills may be the result of other factors present in the environment, leading to a false diagnosis of AGD and unnecessary bathing of fish; and the absence of amoebae in gill sections may be a

result of the fixative used. Thus, there was a need to develop a more rapid, specific and sensitive diagnostic assay, which would not require fish to be sacrificed. It was also considered necessary to identify the best fixative/s for the observation of gill histopathology for the diagnosis of AGD.

Rapid development of AGD has occurred in cages moored amongst cages containing AGD infected fish, while fish moored in cages 75-100 metres away followed the normal pattern of infection (S. Percival, Pers. Comm.). Dr Percival suggested that the “strategic placement of cages on the farms may have potential to reduce the impact of AGD”. This suggests that the numbers of *Paramoeba* in and around infected cages of fish were most likely higher than would normally be present in the open sea. With this in mind, methods were investigated to determine *Paramoeba* numbers in the environment, in order to predict AGD outbreaks in cages at risk of infection, and perhaps identify other targets for non-chemical control of AGD.

6.2 METHODS

6.2.1 Improving diagnosis

Fixative comparison

A small study was carried out to determine the best fixative for histological diagnosis of AGD, to improve the diagnosis of AGD using fixed haematoxylin and eosin (H&E) stained gill material. Gill samples were collected from five fish (as described in Method 2.6.2.) and placed into the following fixatives; sea-water formalin (Appendix 6.1), isotonic formalin (Appendix 6.2), sea-water Davidson’s (Appendix 6.3), isotonic Davidson’s (Appendix 6.4), and Bouin’s (Appendix 6.5) fixatives. Gill samples fixed with sea-water Davidson’s, isotonic Davidson’s and Bouin’s fixatives were transferred to 70% alcohol within 24 hours of fixation. The samples were then processed, sectioned and stained with H&E, and examined for the presence of amoebae, mucus and mucous cells, fusion of lamellae, hyperplasia and other abnormalities or signs of AGD, as described in Method 2.6.2. The fixatives were

compared for each fish sampled on the ability to fix and retain mucus and amoebae; and to preserve the integrity of the gill tissue. Histological examination of gill samples was performed by Dr Judith Handler of the Fish Health Unit, DPIWE, in Launceston, Tasmania.

The gross gill health of the five fish sampled was assessed as described in Method 2.6.1. In addition, smears were taken for IFAT, and the IFAT performed as described in Method 2.6.3.

***Paramoeba* immunofluorescence antibody test (IFAT)**

An immunofluorescence antibody test was developed to detect whole *Paramoeba* in gill smears, using antiserum generated in a rabbit against *Paramoeba* (PA-016) (as used in Chapter 3) and an anti-rabbit FITC conjugated antibody, as described in Method 2.6.3. To optimise the assay, incubation times, temperatures and antibody concentrations were varied to obtain optimal fluorescence in the shortest time period.

To determine the sensitivity of the IFAT compared to the examination of gill histopathology a comparative study was undertaken. A number of farms were asked to routinely submit gill smears in conjunction with gill sections for comparison, over a number of AGD seasons. Smears were taken in one of two ways, either they were taken from live fish and then the fish killed for histological sampling, or the fish were killed and both samples taken from dead fish (as described in Method 2.6.3). Most gill samples were fixed in sea-water Davidson's fixative as requested, but some were fixed in isotonic Davidson's and isotonic formalin. The sections were processed and stained with haematoxylin and eosin and observed for the presence of amoebae and histological signs of AGD. Sections were considered positive for AGD by the presence of typical AGD lesions in the gills, with or without the presence of amoebae. Histological examination of gill samples was performed by Dr Judith Handler. The IFAT was performed as described in Method 2.6.3.

***Paramoeba* field based diagnostic assay**

The initial success of the IFAT in detecting *Paramoeba* in gill smears lead to interest in the development of a field based assay which could be performed at the farms with little sophisticated equipment. Two methods were investigated.

A small number of gill smears known to be positive for *Paramoeba* were screened using antiserum to PA-016 and the immunoperoxidase reagents described in Method 2.7.2. Incubation times, dilutions and temperatures were varied to achieve optimal staining. After immunostaining the smears were examined for dark red/brown stained amoebae against a pink background. All peroxidase stained smears were compared to smears tested by the IFAT.

Multiple gill smears were collected from fish with clinical AGD. *Paramoeba* were detected in the smears using antiserum generated in rabbits against *Paramoeba* (PA-016) and a commercial immunostaining kit (Vectastain ABC-AP Kit - Rabbit IgG, Vector Laboratories, Cat # AK-5001) consisting of a biotinylated anti-rabbit antibody and avidin-alkaline phosphatase conjugate, and a commercial substrate staining kit (Vector Blue Kit, Vector Laboratories, Cat # SK-5300). This method was similar in most aspects to Method 2.7.3. The slides were then counterstained with haematoxylin (Appendix 8.8) and observed under a light microscope for blue *Paramoeba* on a pink background. Incubation times and temperatures were varied to optimise the assay. One smear from each fish was tested with the IFAT to confirm the presence or absence of *Paramoeba*. The smears were examined for the presence of blue stained *Paramoeba* against a pink background. Field assay results were compared to the IFAT to ensure the sensitivity and specificity.

6.2.2 Environmental prevalence

Two methods were investigated to determine the number of *Paramoeba* present in sea-water.

Membrane filtration

The use of membrane filters and fluorescence microscopy for direct counting of aquatic bacteria is well recognised (Hobbie *et al*, 1977; Watson *et al*, 1977). The use of this method was investigated to determine the number of *Paramoeba* present in sea-water. Membrane filtration was undertaken using modifications of the methods used by Hobbie *et al* (1977) and Watson *et al* (1977), and *Paramoeba* detected using the IFAT method, as described in Method 2.6.3.

Sterile sea-water (Appendix 1.2) was seeded with *Paramoeba* sp. 'AGD' (PA-016) to provide positive control samples for development. Sea-water samples were collected from tanks containing fish infected with AGD by cohabitation (see Chapter 4). All samples were fixed with 0.5% (v/v) formalin.

Sea-water samples were tested with and without prefiltration. Samples were prefiltered through 25mm diameter nylon filters with a pore size of 100µm, or 100 µm nylon mesh and 50 µm stainless steel filter prior to testing. Millipore transparent Isopore™ track-etched polycarbonate membranes with pore sizes of 3µm (TSTP 025 00) and 5µm (TMTP 025 00), and a diameter of 25mm were prestained for 24-48 hours in 0.2% irgalan black in 2% (v/v) acetic acid. The dye is available from Ciba-Geigy Corp., Dyestuffs and Chemicals Division, Greensboro, N.C. The stained membranes were rinsed in sterile R.O. water (Appendix 1.1) and used immediately, or air dried before storing. A Millipore black Isopore™ membrane with a pore size of 0.2µm and a diameter of 25mm was also investigated; pore sizes larger than 0.4µm were not available in black (white or transparent membranes cannot be used for immunofluorescence staining due to background fluorescence). Immediately prior to use, dry membranes were wetted with sterile R.O. water and placed into a

Gelman 25mm filter funnel (product number 4203) capable of holding 200ml, which was attached to a glass 250ml side-arm flask. Both the funnel and flask were autoclaved at 121°C for 15 minutes before use.

Seeded sea-water samples were slowly drawn through the membrane by vacuum. The membrane was then rinsed with PBS (Appendix 8.2) and the IFAT performed as described in Method 2.6.3. During antibody incubations the vacuum was removed and the side-arm blocked to allow continued contact of the membrane with the antibodies, and the apparatus placed at 37°C to incubate. To determine the optimum incubation times, antibodies were incubated for periods varying from 15 minutes to one hour. After immunofluorescent staining the damp membranes were placed onto a microscope slide and mounted in alkaline buffered glycerol (Appendix 8.4), and then examined at X100 magnification with a Leitz Dialux 20 UV microscope and an I2 filter pack. At least 10 microscopic fields were observed per membrane for the presence of brightly fluorescing yellow/green cells.

A number of methods were used to reduce the level of background fluorescence. The membranes were stained in 0.003% Evans Blue (Sigma, Cat No. E2129) for one minute, and rinsed in PBS, prior to mounting in alkaline buffered glycerol. The percentage of bovine serum albumin in the primary antibody diluent was increased from 0.1% (v/v) to 5% (v/v), and 1% (v/v) added to PBS used to dilute the secondary antibody. In addition, the IFAT reagents were filtered using a 0.45µm cellulose membrane to remove any bacteria that may have been present.

Analytical flow cytometry

Analytical flow cytometry (AFC) is a technique for the rapid characterisation, quantification and sorting of cells and other particles. This is achieved by simultaneous multiple measurements of light scatter and fluorescence produced by cells as they pass across a laser beam. Measurements include chlorophyll, protein, and DNA levels; immunofluorescent and autofluorescent properties; and size (Burkill, 1987; Shapiro, 1990). Specific antibodies and stains are used differentiate the target organism.

This technique was investigated for detecting and quantifying the number of *Paramoeba* in sea-water samples. The investigation was undertaken with the Australian Environmental Flow Cytometry Group at the Macquarie University in Sydney, under the direction of Mr. Graham Vesey and Dr Nick Ashbolt. Two methods were investigated: detection using *Paramoeba* antiserum to PA-016; and detection using this antiserum and a DNA stain. A Becton Dickson FACScan flow cytometer fitted with an air-cooled 488nm argon ion laser, which can measure three wave-lengths simultaneously, was used in this study.

Samples of natural sea-water (see Appendix 1.1), concentrated by centrifugation at 2,000rpm for 20 minutes, were seeded with *Paramoeba* (PA-016). Concentrated samples were then incubated for 10 minutes at 37°C with the polyclonal antiserum to PA-016 at a dilution of 1:160 in PBS containing 2% bovine serum albumin (BSA). After washing in PBS containing 1% BSA the samples were then incubated for 10 minutes at 37°C with an anti-rabbit FITC conjugate (Silenus Cat No. 984131020), and the samples analysed immediately. Flow cytometric analysis involved the collection of three parameters: forward angle light scatter (FALS), a measurement of size; 90° light scatter (90°LS), a measurement of retractability, internal characteristics and size; and a fluorescent measurement at 525nm (FITC), a measurement of the fluorescence of FITC.

Experiments were then performed to stain the *Paramoeba* with a DNA stain and the polyclonal antiserum simultaneously. The DNA fluorochrome stain (YO-PRO-1 available from Molecular Probes Inc. USA) was used to stain the nuclei and parasomes of the cells. *Paramoeba* were also labelled as above except an anti-rabbit phycoerythrin (PE) labelled conjugate (Calbiochem) was used. This made it possible to have a green and red fluorochrome attached to the cells. Samples of concentrated sea-water were seeded with *Paramoeba* and stained with the polyclonal antibody, followed by the anti-rabbit PE conjugate, and the DNA stain. Samples were analysed immediately using FALS, 90°LS and detection of fluorescence at 509nm and 575nm for the two fluorochromes.

6.3 RESULTS

6.3.1 Improving diagnosis

Fixative comparison

All fixation techniques investigated were considered to be adequate. The fish in this study showed gill damage indicative of moderate to severe AGD, and damage associated with clubbing and necrosis gill syndrome (CNG) (see Clarke *et al*, 1997). The AGD IFAT showed the presence of small to large numbers of *Paramoeba* on the gills. The best fixation was observed with Bouin's, which provided optimum fixation of both the gill tissue and amoebae, allowing detailed observation of the gill tissue to the cellular level and amoebae *in situ*. The next best fixative was sea-water Davidson's, although some damage to the gill at the cellular level was observed. Both Bouin's and sea-water Davidson's fixatives showed the best fixation of free mucus (often containing amoebae), with unattached amoebae being better retained with these fixatives. Sea-water formalin was superior to straight formalin in fixing the amoebae, but did not fix free mucus as well as either Bouin's or Davidson's fixatives. Isotonic Davidson's was more variable, sometimes showing marked leaching of staining characteristics.

Paramoeba immunofluorescence test (IFAT)

The IFAT was successful in detecting *Paramoeba* in gill smears (Figure 6.1). *Paramoeba* fluoresced strongly in the smears and were most often observed within the mucus (Figure 6.2). Mucus did not appear to interfere with antibody binding. The best immunostaining was observed when the incubation times were set at 60 minutes for the anti-*Paramoeba* antibody and 30-45 minutes for the anti-rabbit at 37°C. When the incubation of the anti-*Paramoeba* antibody was reduced to 30 minutes at 37°C staining intensity was reduced, this reduction was also seen when either antibody was incubated at room temperature. Incubation of the anti-rabbit fluoroscein conjugate was not as crucial to staining being optimal at both 30 and 45 minutes at 37°C, while at 1 hour the background fluorescence was marginally higher with the mucus retaining more of the fluoroscein.

A level of sensitivity of the *Paramoeba* IFAT was determined by directly comparing routine gill smears and gill histopathology. A total of 161 gill smears were analysed by IFAT and compared to gill histopathology, Table 6.1 shows the results of this testing. The results show a 96% agreement between the methods. The calculated positive predictive value for the IFAT test was 94% and the negative predictive value was 97%, resulting in a corrected kappa coefficient of 0.91 (statistical analysis courtesy of Douglas-Helders *et al*, 2001). The corrected kappa coefficient was calculated to show the level of agreement between the two methods of analysis; a kappa value of 1 indicates complete agreement, whilst a kappa value of zero indicates no agreement beyond that which is expected by chance. For clinical purposes a kappa value greater than 0.6 indicates a high level of agreement (Saksida *et al*, 1998). Hence the corrected kappa value calculated for this data of 0.91 indicates a very high level of agreement between the two tests.

Table 6.1: Comparison of IFAT and histology in the diagnosis of AGD – data given as the number of tests in agreement or not in agreement.

GILL SMEAR (IFAT)	HISTOLOGY		
	Positive	Negative	Total
Positive	50	4	54
Negative	2	105	107
Total	52	109	161

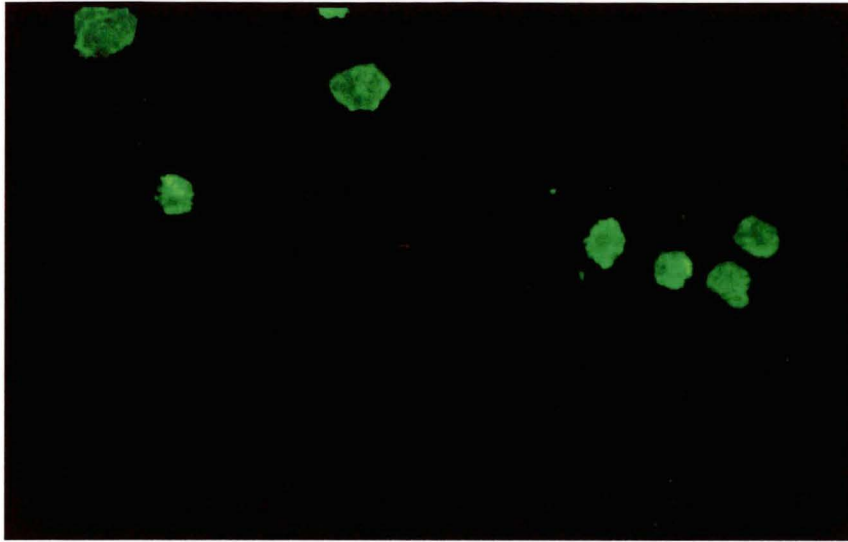


Figure 6.1: Gill smear from an AGD infected Atlantic salmon immunostained by the *Paramoeba* IFAT method, showing highly fluorescent *Paramoeba*. X100 magnification (UV microscopy)

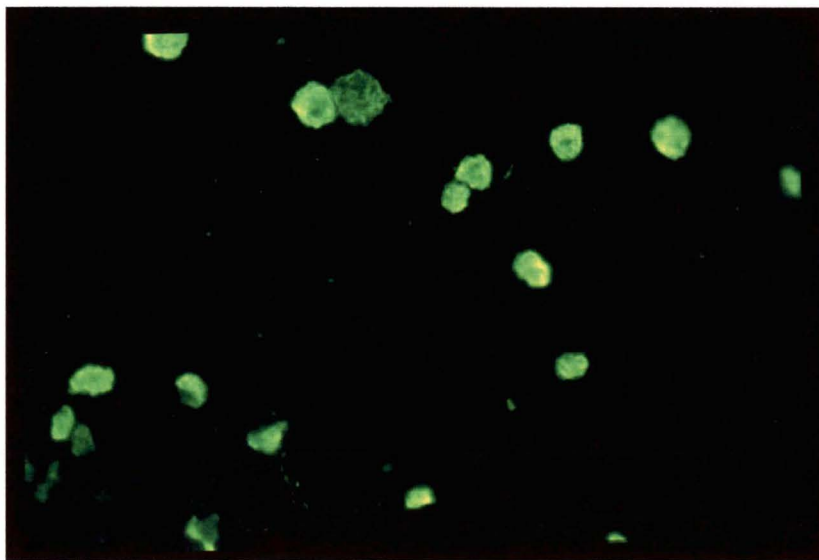


Figure 6.2: Gill smear from an AGD infected fish immunostained by the IFAT, showing highly fluorescent *Paramoeba* attached to mucus (lower left corner). X100 magnification (UV microscopy)

***Paramoeba* field based assay**

The immunoperoxidase technique was not successful as the mucus absorbed much of the counterstain, making it difficult to differentiate the darker staining amoebae.

The immunoalkaline phosphatase staining technique was successful with *Paramoeba* staining blue against a pink background (Figure 6.3). No specific blue staining was observed in negative gill smears (Figure 6.4). A two hour gill smear test was developed to allow farms with a light microscope to undertake on-site diagnosis. Investigation of room temperature incubations did not yield the contrast required, hence staining was undertaken at 37°C in a moist box on a relatively inexpensive slide warmer. A preliminary comparison of the IFAT and the field assay was conducted on 20 smears, with complete concordance of the results.

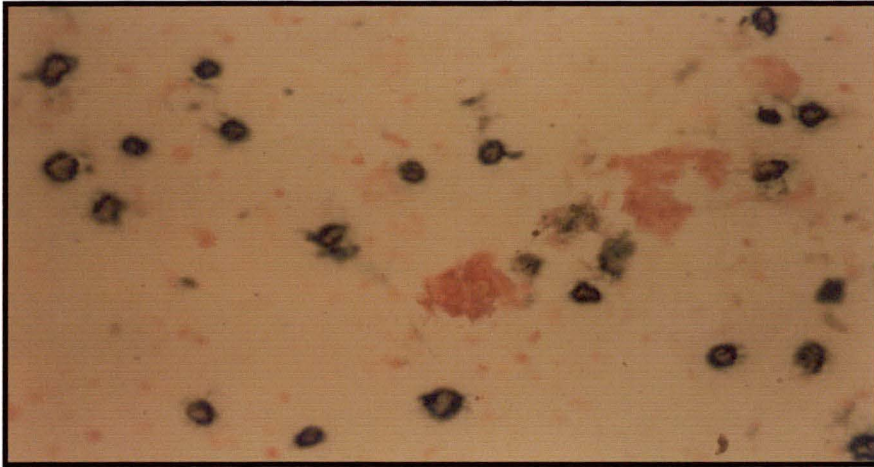


Figure 6.3: Gill smear from an AGD infected Atlantic salmon immunostained by the *Paramoeba* field based assay, showing blue *Paramoeba*. X100 magnification (light microscopy)

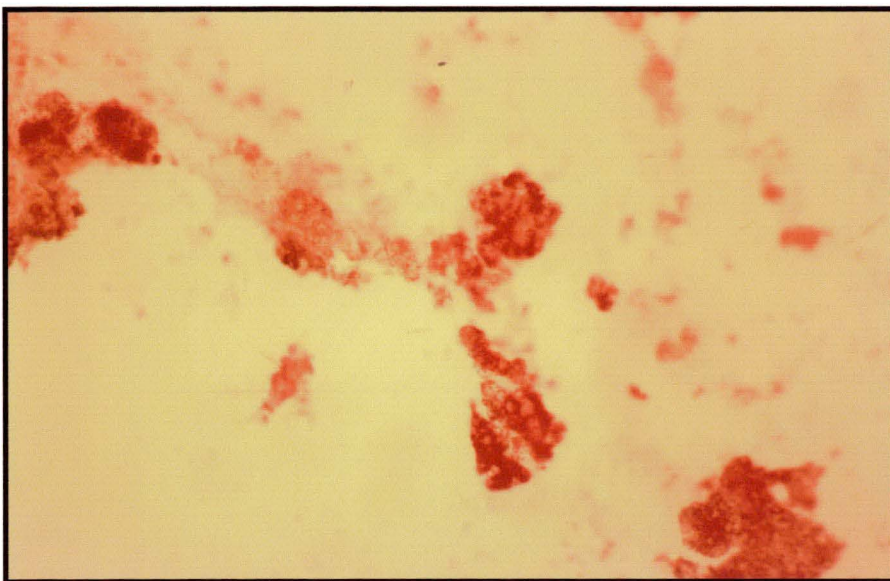


Figure 6.4: Gill smear from an Atlantic salmon not infected with AGD, immunostained by the field-based assay, showing no specific staining. X100 magnification (light microscopy)

6.3.2 Environmental prevalence

Membrane filtration

Filtration of seeded sea-water samples using the 0.2µm filter showed small numbers of positively fluorescing *Paramoeba* on the surface of the membrane, but these were largely obscured as much of the membrane was covered in debris. Prefiltration through two 100µm nylon filters reduced the level of particulate matter on the membranes, however unacceptable levels of debris was still observed. The debris was thought to be a result of the *S. maltophilia*, the bacterial substrate used in the culture of *Paramoeba*. The volume of seeded sea-water able to be filtered through this membrane prior to prefiltration was approximately 25-50ml, after prefiltration it was between 100-120ml. The use of this membrane was abandoned.

The use of the 3µm and 5µm pore size filters and prefiltration through a 100µm nylon filter and a 50µm stainless steel filter improved the volume of sea-water able to be filtered to approximately 300ml. Weakly fluorescing *Paramoeba* were observed on the 3 µm and 5 µm filters of samples seeded with *Paramoeba*. However it was still difficult to estimate *Paramoeba* numbers due to the level of debris on the filters and the 'shattered' appearance of the amoebae. No *Paramoeba* were observed in sea-water samples taken from tanks containing fish infected with AGD, but high levels of debris were also observed on these filters that may have obscured the amoebae.

The use of Evans Blue significantly reduced the background fluorescence. No apparent effect was observed by increasing the BSA concentration in the diluents, or due to the filtration of reagents. The best results were observed with antibody incubation times between 15-30 minutes. However, this method was abandoned due to high levels of debris on the filters that prevented accurate counts of amoebae, and the relatively small volumes of sea-water that could be processed.

Analytical flow cytometry

The use of the polyclonal antiserum to *Paramoeba* and the FITC-conjugate resulted in a high degree of non-specific binding of the antibodies that interfered with analysis and prevented the detection of *Paramoeba*. A high number of debris particles were observed to be fluorescing very brightly. Analysis of an unlabelled concentrated sea-water sample resulted in no fluorescent particles, indicating that the fluorescing particles observed in the *Paramoeba* seeded samples were not due to autofluorescence but were the result of non-specific binding of the antibodies. To determine whether the *Paramoeba* polyclonal antiserum or the FITC-conjugate were responsible for the high background a concentrated sea-water sample was immunostained with the FITC-conjugate alone. Some non-specific binding of the conjugate was detected, but the number of fluorescing particles was considerably less than when both antibodies were used.

Staining with the *Paramoeba* antibody and a phycoerythrin conjugate, simultaneously with a DNA fluorochrome stain enabled the detection of *Paramoeba* in sea-water. The results showed that *Paramoeba* could be detected at high concentrations in sea-water. The limit of detection was 50 *Paramoeba* per ml of sea-water.

6.4 DISCUSSION

Freshwater bathing and improvements in husbandry on the farms, such as reductions in stocking densities and better cleaning of fouled nets, have provided a successful means for the control of AGD, virtually eliminating the devastating losses which had been experienced by farms soon after the commencement of salmon farming in Tasmania (Munday, 1988). Despite the costs involved in freshwater bathing and in the absence of a quick fix chemical treatment, the industry has become resigned to the continued use of freshwater bathing. Competition from larger salmon growing nations has meant that the Tasmanian salmon industry must continue to limit the use of chemotherapeutants in order to maintain its niche market. Hence the emphasis of

research into control methods shifted during the course of these studies to developing methods to improve the management of AGD. Improvements in the diagnosis and detection of the environmental prevalence of *Paramoeba* would thus have important implications in the management of AGD.

6.4.1 Improving diagnosis

Diagnosis of gill damage and AGD by histological examination of chemically fixed gill sections depends greatly on the properties of the fixatives used. These fixatives vary in their ability to fix the gill tissue, mucus, or *Paramoeba in situ*. In AGD, *Paramoeba* are often found to be associated with mucus. Accurate assessment of AGD relies heavily on the observation of *Paramoeba in situ*, and this is especially important since the discovery of the new gill syndrome, clubbing and necrosis gill syndrome (CNG syndrome) with pathological gill changes similar to AGD. Hence the best fixative for AGD must provide the best fixation of mucus and *Paramoeba*. Prior to this study there was no uniform fixative used, and routine submissions to the Fish Health Unit included gills samples fixed in isotonic formalin, Davidson's and less often sea-water Davidson's. A uniform fixative for AGD diagnosis would reduce the subjectivity and variability in the interpretation of histological sections and improve the accuracy of diagnosis.

All the gill fixatives examined were considered adequate, enabling accurate assessment of AGD in the fish sampled for the fixative study. However, these results were influenced by the advanced level of AGD in the fish sampled. Based on past experience, diagnosis of fish with mild or early AGD is more affected by fixative type than diagnosis in heavily infected fish. However a number of differences were observed in the ability of the fixative to fix mucus and amoebae, and the gill tissue. Bouin's fixative gave the best fixation of mucus and *Paramoeba in situ*, it also gave superior fixation of the gill tissue. The use of this fixative in the farms was not considered advisable as Bouin's contains picric acid, a highly toxic chemical requiring special handling and disposal procedures. It was therefore recommended for use only by qualified researchers in special investigations, where its use and disposal

can be controlled. The recommended fixative for the gills of fish with suspected AGD is sea-water Davidson's, a rapid fixative fixing *Paramoeba in situ*, before they fall off the tissue. It is a superior fixative of mucus, which is important as amoebae are often found in large numbers in the mucus, however it can be harsh on gill tissue. Sea-water formalin is the best fixative to examine gill changes at the cellular level. Isotonic formalin and Davidson's were inferior to the same sea-water buffered fixatives.

The application of indirect immunofluorescence antibody tests (IFAT) to detect protozoa in a water and a range of clinical material, including fish is well recognised (De Jonckheere, *et al*, 1974; Chan *et al*, 1993; Adams *et al*, 1995; Marca, *et al*, 1996; Sakida *et al*, 1999). The *Paramoeba* IFAT developed in this study represents the first rapid method of diagnosing AGD. The 96% agreement between histology and IFAT, and the corrected kappa coefficient of 0.91 indicates a very high level of agreement between the two tests. The true sensitivity and specificity could not be calculated in the field because there is no true standard test with which to compare it. The calculated positive predictive value of 94% and negative predictive value of 97% for the IFAT compared to histology suggests that the IFAT is a sensitive and specific assay for the diagnosis of AGD. Of the six discrepant samples noted, four showed the presence of *Paramoeba* in the smears before histological evidence of AGD, indicating the presence of *Paramoeba* in the gills before detectable damage. This result has been confirmed by subsequent routine testing where histological samples have given equivocal results for AGD, but smear results show the presence of *Paramoeba*. Similarly, field observations showed *Paramoeba* were observed in gill smears before gross visible signs of disease were seen in the fish (D. Cameron, T. Lewis and P. Bender, Pers. Comm.). Two other discrepant samples showed histological evidence of AGD and CNG syndrome changes (which both share some similar gill pathological changes), without the presence of *Paramoeba* in the gill smears. This could have been because the primary infection was CNG syndrome with resolving or early AGD, or alternatively that the changes were due to CNG syndrome alone.

The *Paramoeba* IFAT has a number of advantages over routine histological examination: a result can be provided within three hours of receipt by the laboratory, an improvement over histological diagnosis which takes approximately one week for a result; sampling does not require sacrifice of the fish, which is particularly important when sampling expensive harvest sized fish; the process of sampling can be undertaken more often, usually during routine weight checks and requires less hands on time; requires no handling of toxic chemicals; and the test cheaper than histology, as there is no expensive tissue processing time, and a laboratory technician can perform the test.

In practice the most important limitation of the IFAT was the quality of the smear submitted to the laboratory and the transportation of slides. Slides were sometimes submitted with very little mucus present on the surface, and some were transported wet. Close collaboration with the farms led to improvements in sampling technique and transportation of slides. This method is now widely used in Tasmania for confirmation of AGD, as a *de facto* 'gold standard' (Zilberg *et al*, 1999; Douglas-Helders *et al*, 2001).

The field assay was developed in response to the need of some farms to obtain results more rapidly than the IFAT would allow. Industry specifications of the proposed assay required it be rapid; could be performed on routine mucus smears; could be performed by non-scientific personnel; be inexpensive and able to be performed in the field using basic field lab equipment. As most farms possessed a routine light microscope the assay would have to be colourmetric. Two methods were investigated using immunoperoxidase and immunoalkaline phosphatase staining techniques, on gill smears submitted for IFAT. The immunoperoxidase method was unsuccessful, with no contrast being observed between the mucus and *Paramoeba*, the mucus absorbing much of the peroxidase. However the immunoalkaline phosphatase was successful, *Paramoeba* staining blue against a pink background; the mucus did not absorb the alkaline phosphatase enzyme. The assay was optimised to a two hour field assay, requiring only a light microscope and a simple slide warmer to conduct incubations at 37°C. The assay is more expensive than the IFAT and has a more

complicated methodology. For this assay to be sensitive farm staff require training in the performance and interpretation of results. Previous experience training non-scientific farm staff to take bacterial and pathology samples has shown this to be difficult, requiring constant monitoring and retraining. Hence the most likely person to perform field testing would be a field based scientist or veterinarian. Despite the success of this assay it appears unlikely it will become a routine field assay, due to the cost of the reagents, the complexity of the test and the availability of field based scientists.

Since this study was conducted some new methods of diagnosing AGD have been developed. Zilberg *et al* (1999) developed a simple gill smear test using Quick Dip® staining, a rapid haematology stain, for the detection of *Paramoeba*. This test was compared to the IFAT, a 96% agreement between the two tests was observed, with a kappa value of 0.7628, indicating a high level of agreement. The Quick Dip® is not specific for *Paramoeba* however, allowing the identification of other external parasites that may be present in the gill. This may be a disadvantage if other parasites are present on the gills, and thus is open to misinterpretation. Examination of Quick Dip® stained gill smears relies on the observation of blue stained cells, of the size and shape of *Paramoeba* and internal features such as the presence of parasomes. The authors' work validated the use of the IFAT in diagnosis of AGD, suggesting the use of the Quick Dip® as an alternative to IFAT “where a *Paramoeba* spp. with a different serological profile is involved or where the IFAT test is simply unavailable”.

A dot-blot assay has also been developed to allow mass screening of gill samples for *Paramoeba* (Douglas-Helders *et al*, 2001). This assay was also compared the IFAT to determine its sensitivity and specificity, comparing favourably with a corrected kappa value of 0.88. The development of the dot blot assay relied on the use of a *Paramoeba* clone collected during this study (PA-027, see Chapter 4), culture techniques and other methodologies developed during this study. The dot-blot is slightly more sensitive than the IFAT, but is not expected to replace it as a routine diagnostic assay, rather its use has been tailored specifically for the mass screening of fish in future epidemiological studies.

In addition, the IFAT developed in this study has also been used as the primary test used to investigate the survival of *Paramoeba* on dead salmon, demonstrating the importance of the IFAT developed in the present study in the continued research of AGD in Tasmania (Douglas-Helders *et al*, 2000).

Despite the development of the Quick Dip and dot blot assays (Zilberg *et al*, 1999; Douglas-Helders *et al*, 2001) the IFAT remains the *de facto* gold standard for diagnosis of AGD in Tasmania, and now been in use for approximately seven years.

6.4.2 Environmental prevalence

The development of AGD in winter indicates the continual presence of *Paramoeba* in the environment, in numbers high enough to precipitate disease. It is presently not known what factors or environmental conditions affect the numbers of *Paramoeba* in the sea farm environment. The number of *Paramoeba* required to cause disease is also not known. Rapid development of AGD has been observed in uninfected fish in cages moored amongst cages of AGD infected fish, suggesting cross infection (S. Percival, Pers. Comm.). Knowledge of the numbers of *Paramoeba* required to cause disease and the numbers in and around AGD infected cages may allow accurate placement distances of cages to minimise cross infection and ultimately reduce the amount of freshwater bathing required. In addition, knowledge of the factors affecting *Paramoeba* numbers could provide additional targets for control, and may allow the prediction of AGD outbreaks.

While the use of filters for counting bacteria by fluorescence microscopy is a recognised technique (Hobbie *et al*, 1977; Watson *et al*, 1977), estimating *Paramoeba* numbers by filtering sea-water through membranes was not successful due to unacceptable levels of debris being trapped in the membranes. The amount of sea-water able to be filtered was also thought to be too small to provide an adequate assessment of *Paramoeba* numbers. Jones (1988), found only 3.4 *Paramoeba* per litre of sea-water, sampled from a sea-cage with high mortalities due to AGD, none were

found in samples taken from mildly affected cages. However the numbers found on the gills of AGD affected fish were as high as 4.9×10^6 . Rogerson and Laybourn (1991) reported an annual mean of 8300 amoebae per litre in an estuary in Scotland and an occasional summer maximum of 43000 per litre. These results suggest that litres of sea-water may need to be filtered to obtain an accurate assessment of *Paramoeba*. To overcome these problems the use of analytical flow cytometry (AFC) was investigated as a means of detecting and quantifying *Paramoeba* numbers in the environment.

AFC is being used extensively to determine the number of *Cryptosporidia* oocysts and *Giardia* in water samples (Vesey *et al*, 1993 and 1994; Medema *et al*, 1998; Bennet *et al*, 1999; Ferrari *et al*, 2000). It is also being investigated as a tool to study bacteria, protozoa and algae (Burkill, 1992; Edwards *et al*, 1992; Robertson and Button, 1989; Troussellier *et al*, 1993). AFC allows up to 100 litres of concentrated sea-water to be processed without the interference of debris in the concentrated samples (Medema *et al*, 1998; G. Vesey, Pers. Comm.). Preliminary investigation of this method shows that AFC is a suitable process for the detection of *Paramoeba* in sea water, and has the ability to detect cells in sediments. *Paramoeba* have a large amount of DNA that can be readily stained with a suitable fluorochrome. Staining with the DNA stain and the polyclonal antiserum simultaneously enabled detection of *Paramoeba* in seeded sea-water samples. However, due to non-specific binding properties of the antiserum the detection limits were lower than expected.

Conjugation of the polyclonal antiserum to FITC or phycoerythrin would most likely have improved the detection limit (G. Vesey, Pers. Comm.). In flow cytometry highly specific monoclonal antibodies are preferred over polyclonal antibodies such as the one used in this study. Burkill (1992) in his review on the applications of AFC to marine protozoan research states, "that much of the analytical capability of AFC depends upon probes of known specificity". Further investigation of AFC was not undertaken due to the costs involved in the development of the methods, and gaining access to a flow cytometer.

6.5 CONCLUSIONS

Sea-water Davidson's fixative was recommended as the best fixative for routine fixation of gill tissue for diagnosis of AGD by histopathology. Bouin's fixative was the best overall fixative, but its toxicity limits its use to research applications. Therefore, for AGD diagnosis farms were advised to submit samples in sea-water Davidson's. To differentiate AGD from other related gill pathological changes, it was recommended that samples be taken in both sea-water Davidson's and sea-water formalin. Absence of *Paramoeba* in the sea-water Davidson's fixed gill tissue suggests the pathological changes may not be due to AGD. Isotonic fixatives were not recommended for the fixation of gill tissue in sea-reared Atlantic salmon.

The IFAT developed in this study is a sensitive and specific assay, which shows a high level of agreement to gill histopathology. It is now recognised as the *de facto* 'gold standard' for confirmation of AGD (Douglas-Helders *et al*, 2001), and been use by the industry for approximately seven years. The IFAT is currently conducted at the Fish Health Unit, DPIWE, Launceston, Tasmania. The field assay developed in this study proved to be a successful colourmetric assay capable of being conducted in the field, its use as a routine test to monitor AGD is unlikely due to the complex methodology.

Membrane filtration followed by immunofluorescence staining was unsuccessful in determining *Paramoeba* numbers in sea-water. However, analytical flow cytometry shows enormous potential to accurately determine *Paramoeba* numbers in sea-water and sediments, and warrants further investigation.

Chapter 7. Detection of anti-*Paramoeba* antibodies in Atlantic salmon

7.1 INTRODUCTION

Amoebic gill disease in Atlantic salmon in Tasmania is largely a disease of the fish in their first year in the sea. The absence of major epizootics of AGD in salmon experiencing their second year in the sea suggests that the salmon develop a degree of immunity, but this has not been unequivocally demonstrated. Munday *et al* (1990) suggested that the epidemiological and histological features of this disease suggest there is a development of immunity. However, in the present study AGD was observed in harvest sized fish (see Chapter 3), suggesting that this natural immunity is not always protective.

Studies undertaken by Akhlaghi *et al* (1994) demonstrated the presence of anti-*Paramoeba* antibody in the serum of naturally infected salmon previously exposed to *Paramoeba* (35%), in naïve fish after experimental infection with AGD using co-habitation (48%), and also in salmon inoculated with cultured *Paramoeba* (percentage not stated). Bryant *et al* (1995) also demonstrated the ability of naïve fish to produce antibodies to amoebic antigens when immunised with a range of amoebic protein doses. In addition, work undertaken by Lumsden *et al* (1993) compared the serum and gill (mucus) antibody levels to *Flavobacterium branchiophilum*, the causative agent of bacterial gill disease (BGD), supporting the existence of a “specific inducible gill antibody response independent of systemic antibody production”.

Therefore, with these studies in mind it was decided to undertake a small preliminary study to further investigate the anti-*Paramoeba* antibody response in naturally exposed fish, at various stages post-transfer to the sea. The study parameters were expanded to include gill mucus antibody levels. As suggested by Lumsden *et al* (1993); assuming that specific gill antibodies are protective, vaccination against gill diseases may be possible.

7.2 METHODS

7.2.1 Production of *Paramoeba* antibodies in salmon

Naïve Atlantic salmon were immunised with *Paramoeba* antigen to obtain anti-*Paramoeba* antiserum, using methods similar to the ones described by Bryant *et al*, (1995). These antisera were then used to develop an enzyme linked immunosorbent assay (ELISA).

Two *Paramoeba* isolates were used to immunise fish, both having been isolated from AGD infected fish sampled at Dover; PA-016 was isolated in 1992 and PA-027 isolated in 1994. *Paramoeba* were maintained on malt yeast agar plates (Appendix 1.3) seeded with *S. maltophilia* (Appendix 2.1), and routinely subcultured as described in Method 2.1.2. *Paramoeba* antigen was prepared as described in Method 2.5.1. The concentration of protein was determined using the BIORAD Protein Assay Kit (Cat No. 500-0001). The antigen was further diluted with SSW to give a final concentration of 1mg/ml, representing approximately 5,000,000 *Paramoeba* per ml, and then stored at -20°C.

Twenty-five naïve Atlantic salmon smolt, weight range of 60-80g, were maintained in freshwater at 15°C, in a flow through tank system consisting of 50 litre tanks (2-3 fish per tank) with a flow rate of 10L/hr. The fish were fed daily on a mixture of commercial pellets (Gibson) and black worms (*Tubifex* sp.), tanks were cleaned daily prior to feeding. To allow fish to acclimatise to their new surroundings they were maintained for 4 weeks, prior to immunisation. Prior to immunisation blood was sampled from the caudal vein of each fish, and the fish then being immunised with antigen preparations containing 100µg (18 fish), 50µg (5 fish) or 25µg (3 fish) as described in Method 2.5.3. At week 7 the experiment was terminated and as much blood taken as possible. The fish were then perfused with EDTA-saline (Appendix 13.3) to remove the blood from the gills as described in Method 2.10.1; and then the gills removed, weighed and stored at -20°C. Blood was collected into plastic 10ml centrifuge tubes, and processed to separate the serum, as described in Method 2.5.4. Gill (mucus) antibodies were extracted as described in Method 2.10.2.

7.2.2 Serum and mucus collection from naturally infected fish

To determine the presence of gill (mucus) antibody and the relationship between gill antibody and serum antibody, perfused gill tissue and serum were collected from a number of Atlantic salmon naturally exposed to *Paramoeba* in the field; 10 harvest sized fish, exposed to *Paramoeba* for 20 months; 12 triploid ‘pinheads’ (small, wasted fish, with a characteristically small head) exposed for 20 months, and 14 fish exposed for 8 months. Perfused gill tissue and serum were also collected from 18 salmon kept exclusively in freshwater. Following perfusion, gill tissue was weighed and then snap frozen in liquid nitrogen. Serum was separated as described in Method 2.5.4; and mucus collection and antibody extraction performed as described Methods 2.10.1 and 2.10.2.

7.2.3. Development of the ELISA

The *Paramoeba* antigen (PA-016 and PA-027) was prepared as described in Method 2.5.1. The protein concentration was determined and the antigen stored at -20°C. As washing would not eliminate all the bacterial growth substrate, the antigen was expected to contain some *S. maltophilia* contaminants. To ensure that reactivity on the ELISA was due to the presence of anti-*Paramoeba* antibodies, and not anti-*S. maltophilia* antibodies, the sera and mucous extractions were absorbed with *S. maltophilia* prior to testing by ELISA, as described in Appendix 12.

A standard indirect ELISA was developed using the general principals discussed by Kenney (1991), and similar methods to the ones described by Bryant *et al*, 1995. The ELISA was optimised using serum samples obtained from the salmon inoculated with *Paramoeba* antigen, pre-immune sera served as negative controls and post-immune sera as positive controls. The assay was optimised to maximise the difference between the negative and positive control sera, and hence obtain the optimal sensitivity and specificity. Optimisation of the ELISA was determined empirically.

The ELISA was performed on the serum samples and mucus antibody extracts using as described in Method 2.11. To determine if the antiserum contained any residual anti-*S. maltophilia* antibodies, bacterial antigen was prepared from a pure culture of *S. maltophilia*, and a 1ml volume prepared as described for amoeba antigens, Method 2.5.1. This antigen was then coated onto microtitre plates at 10µg/ml and the ELISA performed, using the technique as described in Method 2.11.

7.3 RESULTS

7.3.1 Fish immunisation

The fish were maintained for four weeks prior to immunisation to acclimatise them to the tank system described. Initially the fish were not feeding adequately, and thus to coax them to feed they were fed on black worms exclusively for approximately one week. Commercial pellets were introduced over the next week and for the remainder of the trial they were maintained on pellets supplemented with the black worms. Feeding responses of the fish were variable, with some fish losing weight during the trial. Of the 25 fish immunised with *Paramoeba* antigen 23 survived; 16 immunised with 100µg, 5 with 50µg and 2 with 25µg; both mortalities occurred immediately after the immunisation procedure. No mortalities were observed after the booster immunisation.

7.3.2 Mucus collection

The perfusion procedure described by Lumsden *et al* (1993) was modified to achieve a more rapid and efficient perfusion in our fish. It was found that exposing the heart and detaching the caudal vena cava from the sinus venosus and injecting EDTA-saline into the bulbus arteriosus did not allow rapid perfusion of large fish. The procedure was also lengthy, often causing the death of the fish, thus preventing successful perfusion. Detachment of the tail and perfusing directly into the bulbous arteriosus, or heart, was the most successful method. Inclination of the head prevented

back flow of blood and EDTA-saline onto the gills, a possible route of contamination, and allowed blood to flow freely from the severed caudal vein in the tail. In addition, it was found that the heart must be beating, thus assisting the flow of EDTA-saline and removal of blood.

Perfusion was successful in all of the smaller fish sampled; of the larger fish 7 out of 36 did not perfuse successfully, mainly due to the death of the fish prior to perfusion. The number of larger fish sampled was limited due to the considerable market value of the fish.

7.3.3 ELISA

All the surviving immunised fish produced a detectable level of anti-*Paramoeba* antibodies using the anti-*Paramoeba* ELISA. Low levels of activity to *S. maltophilia* were observed in all fish. To eliminate this reactivity (or cross reactivity) all test samples were absorbed as described in Appendix 12. Absorption of the anti-serum of inoculated fish was successful in removing anti-*S. maltophilia* antibodies.

Optimisation of the ELISA increased the reactivity of the immune sera from a mean of 4 times the pre-immune serum to a mean of 11.5 times the pre-immune sera. The optimum antigen for coating the plates was antigen made from isolate designated PA-016. The results of immunisation of fish with *Paramoeba* antigen are shown in Table 7.1. There were marked differences between fish inoculated with PA-016 antigen and PA-027, with the latter consistently giving much lower OD's on the ELISA. The minimum and maximum O.D. values of each dose show that the anti-*Paramoeba* response varied markedly between individual fish. The least responsive fish had O.D. values 4 times that of the pre-immune sera, the most responsive 17.5 times.

Table 7.1: Optical densities (O.D.) of fish sera, pre-immunisation and post immunisation with *Paramoeba* antigen; comparing the two antigens and three antigen doses.

	Antigen	No. of fish	Antigen Dose*	Mean O.D.	S.D.	Minimum O.D.	Maximum O.D.
Pre immune	None	25	None	0.087	0.002	0.064	0.139
Post immune	PA-016	2	25µg	1.229	0.128	1.031	1.371
	PA-027	2	50µg	0.676	0.078	0.590	0.744
	PA-016	3	50µg	1.193	0.155	0.915	1.447
	PA-027	9	100µg	0.784	0.196	0.358	1.107
	PA-016	7	100µg	1.226	0.182	0.763	1.524

* Total of two doses
SD = standard deviation

Serum and gill mucus samples, from salmon naturally exposed to *Paramoeba*, were tested on the optimised ELISA to determine the anti-*Paramoeba* antibody reactivity. Salmon never exposed to the sea, and consequently *Paramoeba*, were also tested to ensure the specificity of the assay. Three negative control sera, from the pre-immune fish, were included to calculate a cut-off for reactivity. A sample was considered positive if the O.D. was greater than the mean of the three negative control sera, tested in duplicate, plus 2 standard deviations. Serum and mucous samples from the immunised (post-immune) fish were also included, as were a selected number of pre-immune serum.

Table 7.2 shows the results of testing serum and gill mucus samples for anti-*Paramoeba* reactivity. This assay was not optimal, as high backgrounds were observed on the plates reducing the sample to cut-off ratios. However, using the mean of three negative control sera, in duplicate on the plates, plus two standard deviations the results were still clear. The presence of pre-immune and post-immune sera further

validated this assay, as these sera gave the expected negative and positive results. For simplicity the results have been expressed as either positive or negative. All the harvest sized fish, 50% of the triploid pinheads and 57% of the unbathed fish demonstrated the presence of serum anti-*Paramoeba* antibody. Serum anti-*Paramoeba* antibodies were not detected in salmon kept exclusively in freshwater. Gill mucous anti-*Paramoeba* antibodies were not detected in any of the fish. Statistical analysis of this data was not undertaken due to the low number of fish studied and the high backgrounds observed on the plates. Repeat testing could not be performed at the time due to insufficient antigen. Attempts were made at a later date to repeat these results with fresh antigen. The high backgrounds previously experienced were resolved, however retesting of the post-immune serum showed that some samples had lost much of their reactivity, whilst others remained the same. This was thought to be due to the heavy bacterial contamination in many of the sera, and as it was not known to what degree the other samples had deteriorated complete retesting of the serum samples was not performed.

Table 7.2: Comparison of ELISA reactivity of serum and gill mucous samples, against *Paramoeba* antigen, in a number of fish populations.

Fish Population	Exposure	Number Tested*	Serum reactivity		Gill mucus reactivity	
			Positive	Negative	Positive	Negative
Harvest sized	Natural 20 months	10	10 (1.26)	-	-	8 (0.65)
Triploid Pinheads	Natural 20 months	12	6 (1.35)	6 (0.69)	-	8 (0.67)
Unbathed research	Natural 8 months	14	8 (1.36)	6 (0.72)	-	11 (0.62)
Inoculated	Immunised	23	23 (1.98)	-	-	23 (0.52)
Freshwater	None	6	-	6 (0.55)	-	6 (0.57)
	None	12	-	12 (0.69)	-	12 (0.58)
	Pre-immune	12	-	12 (0.62)	ND	ND

Figures in parentheses indicate the mean sample to cut-off ratios

* Perfusion was not always successful resulting in a lower number of mucus samples tested

ND=Not done

7.4. DISCUSSION

Immunisation of salmon was successful in inducing an antibody response in all surviving fish inoculated with *Paramoeba* antigen. The antibody response varied markedly between the fish immunised with PA-016 and PA-027. This difference may be due to differing amounts of actual *Paramoeba* antigen between these two antigen preparations, as the total protein concentration determined for these antigens could not eliminate the degree to which *S. maltophilia* contributed to the antigen concentration. However, given that there was little difference observed between the doses within each antigen, this was not thought to be likely. The most likely explanation was the use of PA-016 antigen in the ELISA, which suggests that there were slight differences between the two *Paramoeba* isolates.

The antibody response also varied between fish and within individual doses and antigens, probably reflecting individual differences in the fish immunised. Some of the immunised fish appeared to be better feeders than others, and while some appeared to either maintain or increase their weight, others appeared to lose weight. It is thought that stress may compromise the immune system of the fish, and this may explain the difference in antibody responses observed between fish inoculated with the same dose.

The antibody response in immunised fish was not proportional to the dose of antigen administered. The two fish immunised with 25µg (total of 2 doses) of protein produced the highest anti-*Paramoeba* antibody response observed in this study, showing that small amounts of protein are sufficient to induce a strong antibody response. These results agree with that of other researchers (Bryant *et al*, 1995; Akhlaghi *et al*, 1996) who reported similar findings.

The seropositivity of fish naturally exposed to *Paramoeba* differed between the groups of fish sampled. These results probably reflect the differences in length of exposure to *Paramoeba*, and the health of the three groups sampled. The seropositivity of the harvest sized fish was 100% in this study, these fish were healthy market quality fish and probably reflects the immune status of most of the larger fish cultivated for commercial sale. Akhlaghi *et al* (1996) reported seropositivity of 35% in naturally infected harvest sized fish, whilst Findlay *et al* (1995) reported 100% seropositivity in infected fish used to infect naïve fish in cohabitation experiments. The lower level of seropositivity found by Akhlaghi *et al* (1996) may be a result of a less sensitive ELISA, as not all fish injected with *Paramoeba* antigen showed detectable antibody by ELISA; or perhaps it is a reflection of a larger sample size. This is in stark contrast to the findings of this study and that of Bryant *et al* (1995) where all fish inoculated produced an immune response.

The lower seropositivity of the triploid ‘pinheads’ that had been in the sea for 20 months (50%) may be a result of the relative poor development of these fish. Whatever causes salmon to develop into ‘pinheads’ may also compromise the immune system. In minor outbreaks of AGD in harvest sized fish, it is often the ‘pinheads’ or smaller fish that show severe signs of AGD (J. Smith, Pers. Comm.), which suggests that these fish are more susceptible to AGD, maybe as a result of lowered immunity or perhaps because the disease overwhelms these fish. The role of ‘pinheads’ as reservoirs of infection requires further investigation.

The unbathed research fish were exposed to *Paramoeba* in the sea for approximately 8 months, these fish had developed signs of mild AGD that did not require bathing. The fish were kept at a considerably lower stocking density than commercial fish, which probably accounts for the low incidence of AGD. The lower level of seropositivity in these unbathed fish (57%) may be a result of the low incidence of AGD in these fish, or due to a shorter length of exposure than the harvest sized fish. Interestingly, Findlay *et al* (1995) found a seropositivity of 100% in fish recently infected with AGD in a cohabitation experiment, and only 68% in previously exposed fish that were re-exposed to AGD by cohabitation. The authors believed this might be an expression of immunological memory. Perhaps this phenomenon explains the lower seropositivity in the unbathed research fish in this study.

None of the naturally exposed or immunised fish showed evidence of anti-*Paramoeba* antibodies in the gill mucus. This finding concurs with those of Akhlaghi *et al* (1994 and 1996) and Findlay *et al* (1995). If these results are to be believed, they suggest that surface antibodies in the gill mucus are not responsible for natural immunity in fish. However no positive controls could be included in the assay to determine if the anti-rainbow trout monoclonal was able to recognise the gill mucus antibodies. Rombout *et al* (1993) found that monoclonals made to carp serum immunoglobulin did not recognise mucus immunoglobulin. Another problem associated with gill mucus antibodies was the use of the extraction cocktail employed by Lumsden *et al* (1993). This group were successful in detecting gill antibodies in freshwater brook trout infected with *Flavobacterium branchiophilum*, the causative agent of bacterial gill disease. In the absence of a positive control, it is not known if the chemical composition of the extraction cocktail was appropriate for gill mucus in marine fish. In a later publication, the extraction cocktail was changed to include the addition of leupeptin, aprotinin and 1 M guanidine. The guanidine was added to reduce enzymatic degradation, but the reasons for the addition of the other chemicals and the change in extraction cocktail were not reported (Lumsden *et al*, 1994). Until the method of extraction is validated and the recognition of mucus antibodies by the rainbow trout monoclonal is proved or disproved, the absence of anti-*Paramoeba* antibodies in gill mucus remains unresolved.

Whether serum antibody levels confer immunity to AGD is not yet proved.

Immunisation of salmon with *Paramoeba* antigen, and passive immunisation of fish with sheep-anti-*Paramoeba* antibodies, did not protect fish from AGD when these fish were challenged by cohabitation with infected fish (Akhlaghi *et al*, 1996).

However, it should be noted that in Akhlaghi's study infection of AGD by cohabitation was extremely rapid, resulting in 100% mortality within 2-4 weeks. The cohabitation trial was conducted using the tanks described in Chapter 4, and using the methods described for Cohabitation trial 1, before improvements were made to reduce rate of infection. Considerable advances have been made to reproduce disease at a much slower rate, more resembling natural development of AGD (see Chapter 4; and

Findlay *et al*, 1995 and 1998). These advances provide a more realistic model of infection for future field trials of vaccines. Studies undertaken by Findlay *et al* (1995) examined the resistance of salmon to reinfection with AGD in tank trials using cohabitation as a means to infect the fish. The authors found that fish previously exposed to AGD showed resistance to reinfection, as demonstrated by a reduction in the number of mucous patches on the gills, and a seropositivity of 68%, which led the authors to suggest that circulating antibody played little if any role in resistance to AGD. Further investigations by Findlay *et al* (1998) demonstrated that fish bathed for the first time in freshwater and then re-exposed to AGD infected fish were not resistant to reinfection, whilst fish given two industry-simulated baths displayed a high level of resistance. This led the authors to suggest that the resistance may be due to the stimulation of the non-immune system by the infection, however this has not been proved. Additionally, these results have yet to be proved in naturally infected fish in the field.

The high backgrounds observed on the plates used to test the naturally infected fish were disappointing, these backgrounds were reduced using a freshly prepared antigen, however bacterial contamination of the serum samples precluded repeat testing. The seroprevalence results reported by Findlay *et al* (1995), using the same *Paramoeba* isolate (PA-016) in their ELISA, concurred with the results presented in this study, providing further evidence of the validity of the data presented here. In hindsight, the bacterial contamination observed in the serum samples could have been avoided by filter sterilizing the serum samples with a 0.22µm filter cartridge, and the inclusion of an anti-bacterial agent such as thiomersal.

7.5 CONCLUSIONS

In this study, naïve fish inoculated with *Paramoeba* antigen produce a strong antibody response, with no detectable difference in response to immunisation with 25, 50 or 100µg of antigen. The ELISA developed in this study initially showed a wide separation of positive and negative serum OD's which was repeatable between runs, however high background levels were observed on the plates used to test the serum from naturally infected fish which could not be resolved within the study period. Despite the high background levels, testing of serum from naturally infected fish proved the existence of circulating antibody to *Paramoeba* in these fish, a finding later supported by the work undertaken by Findlay *et al* (1995).

Specific gill mucus antibodies were not detected in either the immunised or naturally infected fish, these findings being supported by subsequent work (Akhlaghi *et al*, 1996; Findlay *et al*, 1995). The detection of gill mucus antibodies must remain unresolved until the mucus antibody extraction method is validated, and the recognition of gill mucus antibodies by anti-rainbow trout monoclonals is proved.

The detection of anti-*Paramoeba* antibody in the serum of naturally infected fish in this study provides further evidence for the role of *Paramoeba* in AGD. These results demonstrate that naturally exposed Atlantic salmon produce anti-*Paramoeba* antibodies in direct relation to the time spent in the sea; with all harvest sized fish tested being seropositive. Whether serum antibody levels are merely a response to the *Paramoeba*, or whether they confer immunity to AGD, was beyond the scope of this preliminary study. Considerable work has been conducted by Findlay *et al* (1995, 1998) which suggests to the authors that circulating antibody plays little if any role in resistance against AGD, and that resistance may be due to the stimulation of the non-immune system by infection. However, the development of antibodies in experimentally infected fish may not be indicative of antibody production and levels in fish naturally infected with AGD in the field. What factors affect the immune response of fish to *Paramoeba*, when immunity develops and what level of antibody is protective (if they are protective) are all questions that require further elucidation.

More work is required to investigate the antibody responses of fish naturally exposed to AGD in the field before the role of circulating antibodies in the resistance of AGD is ruled out.

Passive and active immunisation has been attempted but was unsuccessful (Akhlaghi *et al*, 1996), however the methods used to expose the fish to AGD were rapid and probably not indicative of field conditions. Further tank and field trials of potential vaccines are warranted. This study has provided little information regarding the feasibility of a vaccine to protect Atlantic salmon smolt against AGD. However, the methods developed during this study may be important tools for additional study of the antibody responses of naturally infected fish.

Final discussion, conclusions and future directions

The results of the present study have not been formally published but have been made available to the salmonid farming industry and to interested aquaculture researchers through: industry based publications (Howard and Carson, 1991, 1992, 1993a, 1993b, 1993c, 1994 and 1995; Howard, Carson and Lewis, 1993), by oral presentation of results at research seminars and industry meetings, and by the participation in the Aquaculture Post Graduate Lecture Series held at the University of Tasmania, in Launceston. The methods developed during this study were also made available to the Fish Health Unit, DPIWE, in Launceston, to enable continued research in to AGD in Tasmania. The results have also been presented for peer review at two national conferences held by the Australian Society of Microbiology and the Australian Institute of Medical Science, and internationally at the 7th International Diseases of Fish And Shellfish Conference, held in Spain. In addition, these results have been widely cited in subsequent literature relating to AGD in salmonids and turbot, indicating the contribution of this study in the research of this significant disease of salmonids in Tasmania.

The results of this study have provided a solid foundation for future studies of AGD in Tasmania. The purpose of this conclusion is to discuss the results of this study, especially in relation to subsequent studies, and to highlight areas for future research based on the results of the present study.

Isolation and verification of the pathogen

At the commencement of this study, no isolates of *Paramoeba* had been successfully maintained in Australia. Marine amoebae belonging to genera other than *Paramoeba* had been isolated from the gills of fish with AGD (P. Statham, Pers. Comm.), but the role of these other marine amoebae in the disease was unclear. Most of the evidence suggesting a pathogenic role for this species in AGD came from histopathological studies, where amoebae identified as *Paramoeba* could be observed associated with

gill damage (Kent *et al*, 1988; Roubal *et al*, 1989; Munday *et al*, 1990; Dyková *et al*, 1998c). In addition, attempts to challenge naïve fish with cultured *Paramoeba* had failed to produce disease (Kent *et al*, 1988; L. Searle, Pers. Comm.). Therefore, it was important to isolate and characterise amoebae from fish with AGD, and to establish the pathogenic role of the cultured amoebae, by their association with gill damage. Only then would it be possible with certainty to undertake pathogenicity trials, determine the effects of inhibitory chemicals and to develop diagnostic assays.

An extensive sampling programme was undertaken of fish with or at risk of AGD that resulted in the preparation of 680 cultures, and 61 successfully subcultured and purified amoeba isolates. This library comprised the protozoan genera *Platyamoeba*, *Vannella*, *Flabellula*, *Heteroamoeba*, *Vexillifera*, *Acanthamoeba* and *Paramoeba*. This finding was later verified by Dyková *et al* (1999), who found species belonging to the genera *Platyamoeba*, *Vannella* and *Flabellula*, accompanying *Paramoeba* in AGD infected turbot.

Of the six *Paramoeba* isolates successfully purified and subcultured, one was classified as *P. eilhardi*. The remaining isolates were consistent with *P. pemaquidensis* as described by Page (1983) and Kent *et al* (1988). Fixed and frozen gill sections from fish with AGD were immunostained with antisera prepared against the amoeba isolates most likely to be the pathogen responsible for AGD. Only with *Paramoeba* sp. ‘AGD’ antiserum were specifically stained amoebae observed associated with gill damage. Very few of the same sections showed specifically stained amoebae when immunostained by the other antisera tested. *Paramoeba* were often observed in large numbers associated with gill damage, frequently lining the damaged secondary lamellae, encrypted in the damaged tissue and penetrating the gill epithelium. These results verified the pathogen associated with AGD as a *Paramoeba* species, and excluded the overt involvement by other amoebae in AGD. The results also demonstrated that other amoeba species were able to colonise the gills of AGD affected fish, but were not present in sufficient numbers to account for the numbers present on the surface of affected gill tissue.

Fixed gill samples from farmed salmonids in New Zealand and France, with similar histopathology to AGD in Tasmania, were tested in the present study by the *Paramoeba* antiserum, showing positive fluorescence. Subsequently, gill samples from an outbreak of AGD in Atlantic salmon in Ireland also tested positive using the *Paramoeba* antiserum (Rodger and McArdle, 1996), but were negative when tested with the other three antisera prepared in this study (Palmer *et al*, 1997). These results all suggested that the *Paramoeba* species responsible for AGD in salmonids of other sea-farming countries was similar if not the same as the species found in Tasmanian salmonids. Recent studies, undertaken by other researchers, have compared some of the isolates collected during the present study to reference strains of *P. pemaquidensis*, two from AGD infected salmonids in other countries, and found 98% sequence similarity (F. Wong, N. Elliot, J. Carson, Pers. Comm.). These results confirm the identity of the *Paramoeba* isolates collected during the present study as *P. pemaquidensis*, and also that the same organism is responsible for AGD of salmonids in other countries.

Leiro *et al* (1998) criticised the use of immunostaining in the present study, suggesting that “it would be difficult to rule out the possibility of cross-reactivity with other free-living amoebae, and because the technique would not distinguish between association and primary cause”. Although the sera used to probe these histology sections were polyclonal, significant investigation was undertaken to ensure the polyclonal antisera were specific, especially in relation to the *Paramoeba* antiserum, which did not cross react to 42 other marine amoeba isolates. Although Douglas – Helders *et al* (2001) found that polyclonal antiserum to *Paramoeba* sp. ‘AGD’ cross-reacted with the closely related *Neoparamoeba aestuarina* and *Pseudoparamoeba pagei*, the likelihood that the *Paramoeba* antiserum was reacting to a related species of marine amoeba is remote, as neither species was isolated in the present study, and both *N. aestuarina* and *P. pagei* are much smaller than *Paramoeba* sp. ‘AGD’. However, the development of a *Paramoeba* sp. ‘AGD’ specific monoclonal would provide the best means of supporting the conclusions made here, but was not attempted in the present study. However, the development of specific *Paramoeba* sp. ‘AGD’ monoclonals warrants further investigation, in addition to adding further evidence of the pathogenic association of *Paramoeba* in the gills, specific

monoclonals may also make a significant contribution to vaccine development, and improve the sensitivity of any future diagnostic assays developed (see Adams *et al*, 1995).

The results of the present study suggest that parasitic isopods, found in the mouth and on the gills of Atlantic salmon, may act as a reservoir of infection or as a vector/transport host transferring *Paramoeba* from infected to uninfected fish. The evidence for this came from the isolation of a significant number of amoebae, including *Paramoeba*, from isopods allowed to walk across culture plates; and from the presence of different genera of amoebae, including *Paramoeba* species, in fixed and frozen isopod sections. Gross examination of the gills undertaken during the sampling period also demonstrated that the numbers of isopods in the mouth and gills increased during the sampling period, coinciding with the development of moderate to severe AGD. These results suggest that amoebae may be commensal organisms of isopods. Subsequent field work showed that infestation with isopods was variable, with little infestation being observed in some seasons, whilst AGD occurs every year. Furthermore AGD could be transmitted from infected fish to naïve fish by cohabitation, without the presence of isopods on the gills. Whilst isopods may not be a primary reservoir of infection, their role as vectors transferring *Paramoeba* from fish to fish is unknown. If the latter role is the only role for isopods in AGD, then the extent of its role in transmission and contribution to the spread and severity of the disease remains to be determined. The ability of isopods to transmit AGD to naïve fish could be easily tested by removing isopods from the gills of infected fish and placing them on the gills of naïve fish and observing for infection, as described by Nylund *et al* (1993) who demonstrated the ability of sea lice to transmit infectious salmon anaemia virus.

Histological examination of gill sections showed a progression of gill damage or irritation, leading to typical AGD lesions and the demonstrated presence of amoebae. Damage or irritation to the gills was observed before the presence of amoebae in the tissue. These findings were in agreements with observations from other researchers (Nowak and Munday, 1994). The findings presented in this study in relation to gill health were incidental; significant work has been performed since to elucidate the

relationship between environmental factors and AGD prevalence (Clark and Nowak, 1999). The authors presented a range of findings one of which was the occurrence of AGD at a minimum temperature of 10.6°C in fish sampled from one farm during summer. This finding supports the occurrence of AGD in winter reported in the present study. Among the other findings reported by Clark and Nowak was the finding that gross diagnosis, when compared to histological diagnosis was unreliable within the lower range, resulting in 31.8% false negatives and 15.9% false positives, a finding that further supports the use of the rapid IFAT developed in the present study. The authors also immunostained gill sections with the *Paramoeba* specific antiserum prepared during the present study, and using the methods developed in the present study, to confirm the histological diagnosis. They found full (100%) agreement between histological diagnosis and immunostaining, confirming the reliability of this methodology.

Despite the considerable investigations undertaken by Clark and Nowak (1999) there has been no study of the prevalence of AGD or *Paramoeba* on the gills of harvest sized fish, or of fish during the winter. During the present study *Paramoeba* sp. 'AGD' were isolated and detected in gill sections taken from harvest sized fish in winter. This finding constituted the first unequivocal evidence of AGD in winter, contradicting anecdotal reports (prior to Clark and Nowak, 1999) that the disease occurred only in the warmer months. Prior to the present study and that of Clark and Nowak (1999) amoebae had been observed on the gills of salmonids in winter when the water temperatures were close to 10°C, without clinical disease (Foster and Percival, 1988; Roubal *et al*, 1989; Munday *et al*, 1990). Evidence of infection in mature fish raises some doubts regarding the development of protective immunity in older fish, and has important implications if the development of a vaccine is being considered. Further study of the presence of *Paramoeba* in fish gills throughout their life in the sea is warranted, using gross gill health, histopathology and IFAT as a means of diagnosing AGD.

Initially the number of *Paramoeba* strains isolated during the sampling period was low compared to the isolation of other amoebae genera. As a *Paramoeba* species was identified as the amoeba associated with AGD, it was important to develop an isolation method that would increase the isolation of *Paramoeba* species. A method previously developed to remove *Paramoeba* from the gills of Atlantic salmon for enumeration (Jones 1988) was adapted to remove amoebae from the gills for culture. This method, combined with the use of malt yeast agar and *Stenotrophomonas maltophilia* as the bacterial growth substrate, was superior to the other methods initially employed isolating many amoebae genera, including *Paramoeba* from the gills of infected fish. Using this method *Paramoeba* sp. 'AGD' was always isolated from the gills of AGD infected fish. The use of this method however, resulted in an increased number of amoeba isolates, many of which were not *Paramoeba*. Methods were then developed to allow rapid identification of *Paramoeba* isolates. During this study the growth characteristics of amoeba isolates were sufficiently different in some genera to allow preliminary identification of some isolates. This was especially true of *Paramoeba* sp. 'AGD', *Flabellula* and cyst forming amoebae. Using the growth characteristics described in this study many marine amoeba isolates were excluded from primary isolation plates as being *Paramoeba* sp. 'AGD'. Isolates with growth characteristics consistent with *Paramoeba* were then examined for the presence of one or more parasomes using either DAPI-staining, phase contrast microscopy or Gomori's trichrome staining to confirm their identity. In almost all cases where *Paramoeba* sp. 'AGD' were identified by their growth characteristics, the presence of one or more parasomes was confirmed by one of the aforementioned techniques. These methods of identification greatly reduced the time spent processing primary isolation plates, and allowed greater confidence in the isolates being used for drug and pathogenicity trials. *P. eilhardi* was unable to be maintained in culture and it was never isolated again in the present study, hence its growth characteristics were not examined. However, its large size would exclude it as being mistaken for *Paramoeba* sp. 'AGD'.

A number of methods were investigated to cryopreserve the amoebae isolated in this study. A cryopreservation technique used successfully for the preservation of a large number of freshwater amoebae (Robinson *et al*, 1990) was adapted for use with marine amoebae. This method was successful in the cryopreservation of most amoeba isolates except *Paramoeba* sp. 'AGD'. A number of other methods were investigated to cryopreserve *Paramoeba* without success. The American Type Culture Collection freezes *Paramoeba* species, which suggests they have a viable method, although the details of this method have not been made available. Further work is required to develop a method to cryopreserve *Paramoeba*, to protect against the long-term selection of laboratory adapted strains, and to act as a back-up if cultures are lost.

The methods to isolate and maintain *Paramoeba* sp. 'AGD' developed in this study have allowed the isolates collected during this study to be maintained in the laboratory to this day. These isolates and newer isolates collected since this study have formed a library of *Paramoeba* isolates, responsible for AGD of salmonids in Tasmania. They have been used by a number of researchers in their respective studies. Akhlaghi *et al* (1994, 1996) used the isolate designated PA-016 (isolated during the present study, as well as methods of culture and preparation of antisera, in his studies of the immunity of fish to AGD and vaccination of fish. *Paramoeba* cultures and reagents were supplied to Findlay *et al* (1995) to assist in the development of an ELISA assay. Douglas-Helders *et al* (2001) also used a *Paramoeba* isolate collected during this study (PA-027), as well as culture and antigen production techniques to produce antiserum for the development of a dot blot assay.

There is no conclusive evidence to suggest that any other pathogen is involved in AGD in salmon or turbot. The presence of bacterial colonies in some gill lesions in AGD affected fish has been noted (Munday *et al*, 1993), and small numbers of bacteria have been observed in gill sections examined by transmission electron microscopy, mostly phagocytised by the gill-attached amoebae (Roubal *et al*, 1989; Dyková *et al*, 1998). A number of protozoa, aside from amoebae, have also been observed on the gills of AGD affected fish, but these findings have not been consistent (Kent *et al*, 1988; Rodger and McArdle, 1996). In Tasmania, extensive

passive surveillance, in addition to the hundreds of gill sections examined in this study, have failed to find any overt bacterial or protozoal infections (aside from *Paramoeba pemaquidensis*) associated with AGD. However, the role of gill-surface dwelling bacteria in the development of AGD has not been fully investigated. Cameron (1993) reported a rapid colonization and proliferation of marine bacteria on the gills of smolt immediately following transfer to the sea, with a steady but slower increase after the initial colonization. The author suggested that the bacterial load might result in the development of conditions favourable for the proliferation of *Paramoeba*. To date no further investigation of the role of bacterial load in AGD development has been reported. If, as suggested by Roubal *et al* (1989), bacteria were found to favour the development of AGD, management strategies could concentrate on the reduction of bacterial flora on the gills of smolt by the use of prophylactic antibiotics or medicated baths. A sequential study of bacterial load and the development AGD is warranted, and as the aetiology of AGD is likely to be complex (see Clark and Nowak, 1999) such a study would need to be conducted at a number of sites, and take into account the numerous environmental factors known to affect the development of AGD. A simpler investigation would be to conduct a field or tank study of fish with and without prophylactic antibiotic treatment (formulated to reduce the number of bacteria on the gills), and monitor the development of AGD in these fish versus bacterial load on the gills.

There have been no reports on the investigation of viral pathogens in the gills of AGD infected fish. Again, extensive passive surveillance in Tasmania has failed to find any histological evidence for a viral agent contributing to AGD, and limited TEM studies on the gills of fish at risk of AGD have not found any viral pathogens present (J. Handler, Pers. Comm.). High titres of infectious haematopoietic necrosis virus have been reported in the gills of fish affected with infectious haematopoietic necrosis (IHN), associated with pathological changes in the gills resembling AGD-like changes (Burke and Grischowsky, 1984). However, IHN has not been reported in Australia (AQIS, 1997). However despite anecdotal evidence, the presence of a virus in the gills of fish with and at risk of AGD has not been fully investigated. Transmission electron microscopy and cell culture studies on gill tissue from fish with and at risk of AGD would either confirm or eliminate the possibility of a virus in the gills contributing to AGD.

Pathogenicity

The isolation of authentic strains of *Paramoeba*, proven to be associated with gill damage, and the development of methods to maintain *Paramoeba in vitro*, allowed pathogenicity trials to be undertaken, in an attempt to fulfill Koch's postulates. Naïve Atlantic salmon and trout were exposed to large numbers of cultured *Paramoeba*, initially in a flow through tank system, then in a recirculation system. Fish were stressed in a number of ways to increase the likelihood of infection, by increasing the temperature of the water, using Zephiran or hydrogen peroxide to damage the gills, and suppressing the immune system with a glucocorticoid. In an attempt to improve the virulence of the *Paramoeba* cultures, fresh isolates were obtained and these grown on normal gill flora. Despite evidence that *Paramoeba* is the cause of AGD, and the numerous methods used to improve the likelihood of infection, Koch's postulates could not be fulfilled. All evidence suggests that the cultured *Paramoeba* used in these challenges was not virulent under the conditions employed. Only with young cultures, grown on normal gill flora was there any evidence of colonisation of the gills, suggesting that there may be more appropriate culture conditions that maintain the virulence of *Paramoeba*. Colonisation of *Paramoeba* was highest in fish that had died, suggesting that the cultured organism were only able to effectively colonise the gills after death, and may lost important attachment abilities. The only clue in the literature to a possible virulence factor was the reported absence of hair-like filaments on the cell surface of cultured *Paramoeba*, a feature present in gill-attached organisms (Kent *et al*, 1988; Roubal *et al*, 1989). Three isolates maintained in monoaxenic culture for up to two years were examined by electronmicroscopy for the presence of these hair-like filaments. All three isolates showed the presence of fine hair-like filaments, a feature until now only observed in gill-attached organisms, and it is thus unlikely that these hairs play an important role in pathogenicity, as the same isolates failed to produce disease in challenge trials. However, it is not known if the hair-like filaments were compromised during culture. If they are involved in attachment, and attachment is important for pathogenicity, then is a role for these hairs. Further study is required to determine if there are any differences between the hair-like filaments on gill attached *Paramoeba* and cultured *Paramoeba*.

Propagation of a virulent *Paramoeba*, able to infect naïve fish, is an important goal for future AGD research, especially if the development of a vaccine is to be explored. A possible culture technique not explored in the present study is the culture of *Paramoeba* in cell culture using fish cells. This method of culture may represent a closer culture environment to the one experienced by *Paramoeba* on the gills, and may result in a virulent organism. Bols *et al* (1994) reported the development of a cell line derived from the gills of rainbow trout, and suggested the use of this cell line for the study of gill diseases in fish. Studies to determine virulence of the cultured *Paramoeba* compared to gill attached organisms are warranted. Significant work has been done on the virulence of the human amoeboid pathogen *Entamoeba histolytica*, especially in relationship to culture conditions, using isoenzyme (zymodeme) patterns to identify pathogenic markers (Mirelman *et al*, 1986; Mirelman, 1987; Gitler and Mirelman, 1987; Mirelman and Chayen, 1990) as discussed by Mills and Golsmid (1995). Similar studies may elucidate virulence markers in *Paramoeba* sp. 'AGD'.

Although AGD was unable to be established using cultured *Paramoeba*, infection was established in cohabitation trials where naïve fish were exposed to fish infected with AGD. Initially the development of AGD was extremely rapid, and overwhelming, resulting in the death of all fish within 14 days. Refinements were made to this technique, by reducing the ratio of infected to uninfected fish, by not heating the water, and using naïve Atlantic salmon instead of rainbow trout. These refinements allowed infection to be maintained for 158 days. Stringent tank husbandry and the introduction of naïve fish to replace dead fish could allow AGD to be maintained in an artificial tank system indefinitely. The development of this model of infection now allows controlled study of the disease, providing researchers with a constant supply of fish without the seasonal constraints that have impeded research in the past. This simple model of infection can also be used to test potential chemotherapeutants and vaccines. Subsequently, this method has been used by Akhlaghi *et al* (1994, 1995) to test the efficacy of active and passively immunised fish to resist infection, and by Findlay *et al* (1995, 1998) in studies of the resistance to AGD in Atlantic salmon.

Identification of amoebicidal chemicals

The isolation of authenticated strains of *Paramoeba* also allowed the *in vitro* testing of potential amoebicides. The growth inhibition and contact inhibition assays developed in this study were found to be effective methods to determine the amoebicidal activity of chemicals and treatments *in vitro*. Thirty-seven chemicals were tested by the growth inhibition assay for their amoebistatic and amoebicidal activity *in vitro*, resulting in 15 chemicals identified as being inhibitory to *Paramoeba* sp. 'AGD' at the concentrations tested. Of these chemicals, mefloquine hydrochloride, pyrimethamine, nitrothiazole, levamisole and fumagillin were highly recommended for testing as medicated feed treatments; and quinine hydrochloride, quinacrine hydrochloride sulphaquinoxaline, narasin and 4-nitroimidazole were recommended as low priorities for field testing. The other four chemicals were not recommended based on their toxicity or inability to be readily absorbed by the gut.

Of the amoebicidal chemicals identified only fumagillin, quinacrine hydrochloride, levamisole and narasin have been tested, as feed medications to treat AGD (Alexander, 1991; Cameron, 1992). Only narasin and quinacrine hydrochloride reduced the level of infection, although quinacrine was found to accumulate in the tissue of fish turning it a bright yellow, eliminating this chemical as a potential treatment for AGD; and some palatability problems were reported with narasin medicated feed (Alexander, 1991 and Cameron, 1992). Fumagillin was found to be highly toxic to the fish in the field test conducted by Alexander (1991); and it should be noted that when medicated the fish were already infected with AGD, casting some doubt on the reliability of this trial. However, there have been a number of reports of successful treatment with fumagillin of various myxosporean and microsporean pathogens of salmonids (Kent and Dawe, 1994; Higgins and Kent, 1996; le Gouvello *et al*, 1999; Speare *et al*, 1999). A synthetic analogue of fumagillin (TNP-470) has also been shown to be effective in controlling two microsporidian pathogens of salmon (Higgins *et al*, 1998). The success of these treatments, without the associated mortalities observed by Alexander (1991), appears to be due to the use of much lower fumagillin dose rates, and a reduction in the duration of the treatments. Hence, further investigation of fumagillin, and the synthetic analogue TNP-470, is required before it

can be ruled out as a potential treatment for AGD in the field. The remaining amoebicidal chemicals identified by the growth inhibition assay have yet to be tested in the field.

Thirteen chemicals or treatments were tested for their amoebicidal activity *in vitro* by the contact inhibition assay, developed to determine the amoebicidal activity of a chemical or treatment in a short-term aqueous exposure. This assay was used to identify chemicals that could potentially be used in a bath to treat fish with AGD. The results of freshwater testing established *in vitro* that freshwater is lethal to *Paramoeba* within two hours. Prior to this research the lethal effects of freshwater had been postulated, but not confirmed. Of the 12 remaining chemicals or treatments, eight were found to be significantly amoebicidal to *Paramoeba* when exposed for four hours. Hydrogen peroxide was identified as the most promising alternative treatment identified by this study, being able to totally inactivate *Paramoeba* at 100 ppm and an exposure time of 30 minutes. Of the other chemicals or treatments, pyrimethamine, 8-hydroxyquinoline and 5-chloro-7-iodo-8-hydroxyquinoline were recommended for field testing as medicated bath treatments, and further investigation of chloramine-T was also recommended, despite previous reports of its failure to treat AGD (Munday *et al*, 1993). The remaining chemicals or treatment were not recommended based on their toxicity, inability to produce the amoebicidal activity observed *in vitro* in the field, and past testing in the field.

Field testing of hydrogen peroxide was commenced by another researcher soon after the results of this study were made available to industry. The results of testing hydrogen peroxide were disappointing, with concentrations of between 100-300 ppm failing to control developing AGD (Cameron 1994c). Additionally, hydrogen peroxide was found to have a narrow margin of safety due to the temperature dependent nature of its toxicity to Atlantic salmon (Cameron, 1994c). Further investigation of hydrogen peroxide was thus abandoned. However hydrogen peroxide has been described to treat a number of diseases of salmonids, particularly as a delousing treatment for sea-lice infected Atlantic salmon, and used at much higher concentrations than were tested in Tasmania (Thomassen, 1992; Bruno, 1992a; Speare and Arsenault, 1997; Lumsden *et al*, 1998). This suggests that hydrogen

peroxide may still be a useful chemical to treat AGD in countries with lower water temperatures than those experienced by Tasmania. Pyrimethamine, and the hydroxyquinolines have yet to be tested in the field as bath medications.

Recently, levamisole added to freshwater baths was associated with a significant decrease in the mortality of fish exposed to AGD, but no effect was observed with oral supplementation with levamisole (Zilberg *et al*, 2000). These trials were conducted in tanks, and fish infected by cohabitation using methods similar to the ones already described in the present study. The authors suggested that this response was due to enhancement of the non-specific immune system, however they failed to consider the possibility that levamisole could have been amoebicidal. The amoebicidal effects of short-term exposure of levamisole to *Paramoeba* sp. 'AGD' are unknown, as this chemical was not tested in the present study by the contact inhibition assay. The investigation of the amoebicidal effects of levamisole on *Paramoeba in vitro* are warranted to rule out the possibility that the decrease in mortality in AGD infected fish was due to the amoebicidal effects of this chemical rather than due to the enhancement of the non-specific immune system. However, field trials of levamisole added to freshwater baths failed to offer any increased protection to AGD (Clark and Nowak, 1999). Further field trials of levamisole have been conducted by other researchers, which may confirm or eliminate the use of this chemical in the treatment of AGD, the results of which have yet to be published.

Further investigation of the amoebicidal chemicals identified in the present study is warranted. In the past, most of the chemical trials were conducted in the field, where problems with the level of infection and timing of the treatments were observed. The development of a model of infection using cohabitation now allows preliminary testing to be undertaken in tanks, where the timing and level of infection can be more closely monitored and controlled, eliminating treatments that do not work before costly field trials are undertaken. The amoebicidal chemicals recommended are unlikely to emulate the therapeutic effects of freshwater bathing, which is thought to remove the mucus from the gills, restoring osmotic balance to affected fish (Jones, 1988; Munday *et al*, 1990). Feed and bath medications may only be effective when used prophylactically to prevent or control early stages of AGD, when mucous patches are absent or very few in number.

Unfortunately, it is now unlikely that these chemicals will not be investigated further in Tasmania. This is largely due to the disappointing results of the field trials conducted to date, particularly hydrogen peroxide, and a shift in attitude within the Tasmanian industry to the use of chemicals to control disease. Increased competition from larger salmon growing countries means that the Tasmanian salmonid industry must continue to limit its use of chemicals to maintain the its niche markets established on the basis of its relatively chemical-free status. However the additional chemicals identified in this study may be of specific value occasionally when freshwater is not available, thus extending the range of treatments for AGD. Furthermore, these results may be of particular relevance to other countries where AGD occurs, such as Ireland, where freshwater bathing is not practical (Rodger and McArdle, 1996; Palmer *et al*, 1997).

Improving diagnosis of AGD

During the course of this study the emphasis on methods to control AGD shifted from the use of chemicals to the development of methods to control or predict AGD. Prior to this study a definitive diagnosis of AGD was achieved by examining chemically fixed gill sections and observing them for the presence of gill damage and amoebae, consistent with AGD. Although an accurate assessment of gill health, this method required the sacrifice of sampled fish, took a minimum of one week for results and was costly. An additional problem with histological examination of gill tissue was that amoebae were not always observed in gill sections, and that AGD-like gill changes might have been the result of other factors present in the environment. Using the antiserum to a *Paramoeba* sp. 'AGD' isolate prepared during the immunostaining portion of this study, a sensitive and specific immunofluorescent antibody test (IFAT) was developed to detect *Paramoeba* sp. 'AGD' in gill smears. Comparison of the IFAT and gill histopathology showed a 96% agreement between the two methods and a corrected kappa value of 0.91, indicating a very high level of agreement between the two assays. The IFAT developed in this study represents the first rapid method of diagnosing AGD. The IFAT has many advantages over histopathology, the main ones being the speed at which results are available, it does not require sacrifice of the fish

and is considerably less costly than histopathology. This IFAT is now recognised as the *de facto* gold standard for confirmation of AGD (Douglas-Helders *et al*, 2001) and has been used by the industry for approximately seven years. It is currently conducted at the Fish Health Unit, DPIWE, in Launceston, Tasmania.

In response to the needs of industry, a field assay was developed to allow some farms to obtain results more rapidly than the IFAT would allow. A colourmetric assay was developed to detect *Paramoeba* sp. ‘AGD’ in gill smears in the field. This assay compared favorably with the IFAT, but was more complex and costly than the IFAT. For technical reasons, this assay is unlikely to be used by farm staff as a routine test to monitor AGD in the field, but the methodology does not preclude its use by scientific staff in the field when rapid results are required and fluorescent microscopes are unavailable.

Since the present study was completed two assays have been developed to diagnose AGD. Zilberg *et al* (1999) developed a simple gill smear test using Quick Dip® staining, a rapid haematology stain, for the detection of *Paramoeba*. This test was compared to the IFAT; a 96% agreement between the two tests was observed, with a kappa value of 0.7628, indicating a high level of agreement. The Quick Dip® is not specific for *Paramoeba* however, allowing the identification of other external parasites that may be present in the gill. This may be a disadvantage if other parasites are present on the gills, and thus is open to misinterpretation. Examination of Quick Dip® stained gill smears relies on the observation of blue stained cells, of the size and shape of *Paramoeba* and internal features such as the presence of parasomes. The authors’ work validated the use of the IFAT in diagnosis of AGD, suggesting the use of the Quick Dip® as an alternative to IFAT “where a *Paramoeba* spp. with a different serological profile is involved or where the IFAT test is simply unavailable”.

A dot-blot assay has also been recently developed to allow mass screening of gill samples for *Paramoeba* (Douglas-Helders *et al*, 2001). The dot blot assay detects *Paramoeba* antigens present in gill mucus, the collection of which is also not lethal to the fish being sampled. This assay was also compared the IFAT to determine its sensitivity and specificity, comparing favorably with a corrected kappa value of 0.88.

The development of the dot blot assay relied on the use of a *Paramoeba* clone collected during this study (PA-027), and also the culture techniques and other methodologies developed during the present study. The dot-blot is slightly more sensitive than the IFAT, but is not expected to replace it as a routine diagnostic assay due to its complex methodology, rather its use has been tailored specifically for the mass screening of fish in future epidemiological studies.

Neither the Quick Dip® or the dot blot appears to have diminished the importance of the IFAT in the diagnosis of AGD. However, the Quick Dip® offers some added advantages over the field assay developed in the present study. The use of the IFAT as a comparative assay in the development of these two new assays, and the use of IFAT in the investigation of the survival of *Paramoeba* on dead fish, demonstrates the importance of the IFAT in the continued research of AGD in Tasmania.

To further improve the diagnosis of AGD a small study of tissue fixatives was undertaken to determine the most appropriate fixative for gill tissue in the diagnosis of AGD. Prior to this study no uniform fixative was being used by the farms to fix gill tissue. As a result of the present study, sea-water Davidson's fixative was recommended as the best fixative for routine fixation of gill tissue for the diagnosis of AGD by histopathology, and therefore farms were advised to submit gill samples in this fixative for AGD diagnosis. Bouin's fixative was the best overall fixative, but its toxicity limits its use to research applications. Sea-water formalin was found to be the best fixative to examine gill tissue at the cellular level, and hence to differentiate AGD from other related gill pathological changes, farms were advised to submit gill samples in sea-water Davidson's and sea-water formalin. Isotonic fixatives were not recommended for the fixation of gill tissue from sea-reared Atlantic salmon. The results of this study have led to the uniform use of sea-water Davidson's fixative by farms for the routine fixation of gill tissue for the diagnosis of AGD.

Presence of *Paramoeba* in the environment

The development of AGD in winter indicated the continual presence of *Paramoeba* in the environment, in numbers high enough to precipitate disease, and also indicated that AGD could occur in lower water temperatures than was previously thought. Rapid development of AGD in uninfected fish in cages moored amongst cages of infected fish (S. Percival, Pers. Comm.), suggested that numbers of *Paramoeba* in and around infected cages of fish were most likely higher than would normally be present in the open sea. With this in mind, two methods were investigated to determine the number of *Paramoeba* present in sea-water. It was thought that knowledge of the numbers of *Paramoeba* in the open sea, and the numbers required to cause infection, could allow accurate placement of cages to minimize cross infection. In addition, the factors affecting *Paramoeba* numbers could be determined to predict the likelihood of AGD outbreaks. Membrane filtration was not successful in determining the numbers of *Paramoeba* in the environment, however the use of analytical flow cytometry (AFC) showed enormous potential to accurately determine *Paramoeba* numbers in sea-water and sediments. In seeded samples, *Paramoeba* were probed with the polyclonal antiserum used in the immunostaining of gill sections and IFAT assay, and then an anti-rabbit conjugated to a fluorochrome, the *Paramoeba* were also simultaneously stained with a DNA fluorochrome stain. Specifically stained cells were quantified by flow cytometry based on their staining characteristics and size. The detection limits of AFC using this method was lower than expected, 50 *Paramoeba* per ml of sea-water, due to non-specific binding properties of the antiserum. Further investigation of this method was not undertaken in the present study, due to the costs involved in the development of the method, and difficulty in gaining access to a flow cytometer. However, further investigation of this method is warranted to determine if a relationship between *Paramoeba* numbers in the water column and AGD outbreaks are related. Conjugation of the polyclonal antiserum to FITC or phycoerythrin would most likely improve the detection limits (G. Vesey, Pers. Comm.). The detection limit would be further improved if a *Paramoeba* sp. 'AGD' specific monoclonal was available, also conjugated to a fluorochrome – demonstrating yet again the importance of the development of a specific monoclonal.

Paramoeba appear to rapidly proliferate on the gills of fish. Perhaps the *Paramoeba* colonise an intermediary host or surface before colonising the gills of the fish, such as macroalgae or nets. *Paramoeba* is one of the most common amoebae found on species of macroalgae (Rogerson, 1991). Rogerson reported that amoeba species on two types of macroalgae were most abundant in the summer months, where numbers up to 23 amoebae per cm² of algal surface were recorded. *Paramoeba* has been observed on the nets of sea-cages containing AGD infected salmon (Jones, 1988). It is also known that *Paramoeba* will not grow or multiply in suspension but require a solid substrate for *Paramoeba* growth (Martin, 1985). Recently, Douglas-Helders (2000) established that *Paramoeba* from dead AGD infected fish could colonise the gills of previously uninfected dead fish, indicating that AGD mortalities present in sea-cages might be an important reservoir of infection. Further work is required to elucidate if these solid substrates are potential reservoirs of infection in the field. The IFAT may be a simple method to determine if *Paramoeba* are present on the surface of macroalgae and nets.

Detection of anti-*Paramoeba* antibodies in Atlantic salmon

Amoebic gill disease in Tasmania is largely a disease of the fish in their first year in the sea. Although AGD was reported in harvest-sized fish in the present study, major epizootics of AGD in salmon experiencing their second year in the sea are not regularly reported. This evidence suggests that salmon develop a degree of immunity, but this has not been unequivocally demonstrated. Akhlaghi *et al* (1994) reported the presence of anti-*Paramoeba* antibodies in 35% of naturally infected salmon previously exposed to *Paramoeba*. The low incidence of detectable serum antibody in these fish suggested that circulating antibody plays little role in resistance to AGD. However, it was not known if gill mucus antibodies to *Paramoeba* played a role in the observed resistance to AGD. In the present study the detection of anti-*Paramoeba* antibodies was extended to include the detection of specific antibodies in gill mucus, using a technique developed by Lumsden *et al* (1993). If anti-*Paramoeba* antibodies were detected in gill mucus, and assuming that specific antibodies are protective, then vaccination against gill diseases may be possible.

Using methods developed by Bryant *et al* (1995) naïve Atlantic salmon smolt were immunised with *Paramoeba* antigen to produce specific antibodies for use in the development of an ELISA. This technique was successful with all immunised fish producing anti-*Paramoeba* antibodies. An ELISA was then developed to maximise the difference between serum samples positive for anti-*Paramoeba* antibodies and serum samples from naïve fish not exposed to *Paramoeba*. Serum and gill mucus were then collected from a variety of populations of naturally infected salmon, and from naïve fish with no exposure to *Paramoeba*. Gill mucus antibodies were extracted using the methods described by Lumsden *et al* (1993). Serum antibody was detected in all harvest-sized fish exposed to *Paramoeba* for 20 months, and in 57% of fish with only 8 months exposure, but not in fish with no exposure to *Paramoeba*. Interestingly only 50% of ‘pinheads’ (undersized, emaciated fish), with 20 months exposure were positive for anti-*Paramoeba* antibodies. Gill mucous antibodies were not detected in any of the fish. Although some problems were associated with the ELISA during the testing of these samples, similar results have since been reported by Findlay *et al* (1995). The detection of specific antibody in all harvest-sized fish in this study and that of Findlay *et al* (1995) conflicts with Akhlaghi’s earlier report of a 35% serum positivity.

The absence of anti-*Paramoeba* antibodies in gill mucus has also been reported in subsequent studies (Akhlaghi *et al*, 1996; Findlay *et al*, 1995). If these results are to be believed, they suggest that surface antibodies in the gill mucus are not responsible for natural immunity to AGD. However, the extraction ‘cocktail’ used in the present study, and also in the studies undertaken by Akhlaghi *et al* (1996) and Findlay *et al* (1995), has not been validated for use in Atlantic salmon mucus. Lumsden *et al* (1993) used this cocktail successfully to extract antibodies to *Flavobacterium branhiophilum* (the causative agent of bacterial gill disease) from freshwater brook trout. In the absence of a positive control, it is not known if the chemical composition of the extraction cocktail is appropriate for the gill mucus of marine fish. In addition, it is not known if the anti-rainbow trout monoclonal used in the ELISA was able to detect gill mucus antibodies. Therefore, until the extraction method is validated, and the detection of gill mucus antibodies by the monoclonal used in the ELISA proved or disproved, the absence or presence of anti-*Paramoeba* antibodies in gill mucus must remain unresolved.

The results of the present study indicate that circulating antibody may play a role in the resistance to AGD shown by fish in their second year in the sea. The lower seropositivity in 'pinheads' may be a result of the poor development of these fish, or an indication of a compromised immune system. Minor outbreaks of AGD have been reported in harvest-sized fish, with the 'pinheads' being most affected (J. Smith, Pers. Comm.), suggesting that these fish are more susceptible and may be a reservoir of infection in larger fish. The incidence of AGD and serum antibody levels of 'pinheads' warrants further investigation.

Whether serum antibody levels confer immunity to AGD is not yet resolved. Immunisation of salmon with *Paramoeba* antigen, and passive immunization of fish with sheep-anti-*Paramoeba* antibodies, did not protect fish from AGD when these fish were challenged by cohabitation with infected fish (Akhlaghi *et al*, 1996). Although the cohabitation trial was conducted before improvements had been made to reduce the rate of infection, thus was extremely rapid and not indicative of natural infection. Findlay *et al* (1995) found that fish previously exposed to AGD showed resistance to reinfection, as demonstrated by a reduction in the number of mucous patches on the gills, and a seropositivity of only 68%, which led the authors to suggest that circulating antibody played little if any role in resistance to AGD. Further work conducted by Findlay *et al* (1998) found that fish given two industry-simulated freshwater baths displayed a high level of resistance to AGD in tank trials, leading the authors to suggest that resistance may be due to the stimulation of the non-specific immune system, however this has not been proved. Additionally, these results have yet to be proved in naturally infected fish in the field.

Despite the work conducted in the present study, and that of other researchers (Akhlaghi *et al*, 1994, 1996; Findlay *et al*, 1995, 1998), the nature of the observed immunity of salmon experiencing their second year in the sea remains unresolved. The factors affecting the immune response of fish to *Paramoeba*, when immunity develops in naturally infected fish, and what levels of antibody are protective (if any), are all questions that require further elucidation. A prospective study of serum antibody levels in Atlantic salmon throughout their duration of their life in the sea is

warranted, and combined with gill histopathology, IFAT and freshwater treatment details, may resolve the issue of antibody related immunity to AGD. Whether gill mucous antibodies to *Paramoeba* exist also requires resolution. However, as the gill mucus collection method described by Lumsden (1993) requires sacrifice of the fish, this limits the number of larger fish that can be sampled, limiting any future epidemiological studies, and thus a non-lethal method of mucus collection is required. In addition, the active and passive immunisation studies as performed by Akhlaghi *et al* (1994, 1996) require repeating in tank trials where the development of AGD is slower and more controlled development of AGD.

Appendices

The methods and culture recipes supplied were either developed empirically or taken from established methods. Wherever possible the reference is cited. Selected methods and culture recipes were obtained from the Animal Health Laboratory, DPIWE and will be referenced accordingly.

1. Culture media

1.1 Natural and sterile sea-water (SSW)

Natural sea-water 33-35‰ collected offshore, away from boating, stormwater or sewage pollution, was filtered through a 5µm and 0.45µm filter system (Sartorius). For methods requiring sterile sea-water, the filtered sea-water was dispensed in 500ml volumes and autoclaved at 121°C for 15-20 minutes, and then stored at 4°C.

1.2 Reverse osmosis (R.O.) water

Reverse osmosis water was used for all reagent and culture medium preparation. Water had a conductivity of 0.4µSiemens (2.5 mega ohms). For those methods requiring sterile R.O. water, 500ml volumes were autoclaved at 121°C for 15-20 minutes, and then stored at room temperature.

1.3 Malt yeast agar (MYA) (Adapted from Page, 1983)

Non-nutrient agar (Difco Laboratories, 0140-01)	20g*
Malt extract (Oxoid, L39)	0.1g
Yeast extract (Oxoid, L21)	0.1g
Natural sea-water, 0.45µm pre-filtered	750ml
R.O. water	250ml
Sterile pimaricin suspension (Sigma P-0440) 2.5% w/v	500µl

*25g/L was used for 145 and 245mm² petri dishes or bioassay plates

All ingredients except for the pimaricin were mixed and then autoclaved at 121°C for 30 minutes. The medium was then cooled to 70°C, the pimaricin added aseptically and the plates poured. The plates were then dried in a laminar flow cabinet for 1 hour. Plates were stored at 4°C for up to 8 weeks in sealed plastic bags.

1.4 Freshwater agar (FWA)

Non-nutrient agar (Difco Laboratories, 0140-01)	20g
R.O. water	1000ml
Sterile pimaricin suspension (Sigma P-0440) 2.5% w/v	500µl

All ingredients except for the pimaricin were mixed and then autoclaved at 121°C for 30 minutes. The medium was then cooled to 70°C, the pimaricin added aseptically and the plates poured. The plates were then dried in a laminar flow cabinet for 1 hour. Plates were stored at 4°C for up to 8 weeks in sealed plastic bags.

1.5 Sea-water agar (SWA)

Non-nutrient agar (Difco Laboratories, 0140-01)	20g
Natural sea-water, 0.45µm pre-filtered	900ml
R.O. water	100ml
Sterile pimaricin suspension (Sigma P-0440) 2.5% w/v	500µl

All ingredients except for the pimaricin were mixed and then autoclaved at 121°C for 30 minutes. The medium was then cooled to 70°C, the pimaricin added aseptically and the plates poured. The plates were then dried in a laminar flow cabinet for 1 hour. Plates were stored at 4°C for up to 8 weeks in sealed plastic bags

1.6 **Blood agar** (method supplied DPIWE)

Blood Agar Base No. 2 (Oxoid, CM271)	16g
R.O. water	400ml

The medium was autoclaved at 121°C for 15 minutes and cooled to 50°C. To the molten base sterile defibrinated sheep's blood was added, mixed and then poured as plates. The plates were then dried in a laminar flow cabinet for 1 hour. Plates were stored at 4°C for up to 8 weeks in sealed plastic bags

1.7 **Marine Ordal's agar/medium**

(Adapted from Anacker and Ordal, 1959)

Bacto-peptone (Difco Laboratories, 0118-01)	0.5g
Yeast extract (Oxoid, L21)	0.5g
Sodium acetate	0.2g
LabLemco (Oxoid, LP029B)	0.2g
Sodium pyruvate	0.1g
Natural sea-water	900ml
R.O. water	100ml

All ingredients were mixed and then autoclaved at 121°C for 20 minutes. The medium was then cooled to room temperature. Liquid medium was stored at 4°C for up to 8 weeks.

Agar plates were made by adding 11g of non-nutrient agar to the above ingredients, and autoclaving as above. The medium was then cooled to 50°C and the plates poured. The plates were then dried in a laminar flow cabinet for 1 hour. Plates were stored at 4°C for up to 8 weeks in sealed plastic bags

1.8 Growth inhibition agar (Page, 1983)

Non-nutrient agar (Difco Laboratories, 0140-01)	0.8g
Natural sea-water 75%	22.5ml
Malt extract (Oxoid, L39)	0.025g
Yeast extract (Oxoid, L21)	0.025g

Ingredients were mixed in a 100ml disposable polycarbonate bottle and autoclaved at 121°C for 30 minutes. One bottle of agar was prepared for each dilution of each chemical tested in the growth inhibition assay (Method 2.9.3).

2. Bacterial culture substrates

2.1 Live *Escherichia coli* or *Stenotrophomonas maltophilia* suspension

Nutrient Broth No. 2 (Oxoid, CM67)	25g
R.O. water	1 litre

E. coli/*St. maltophilia* cultured on blood agar (Appendix 1.6)

E. coli or *Stenotrophomonas maltophilia* were grown in one litre volumes of Nutrient Broth No. 2 in a two litre flask. The flasks inoculated from overnight plate cultures of the bacteria, were incubated at 37°C on an orbital shaker for 48-72 hours. The cells were harvested by centrifugation at 3,700g and washed 3 times with sterile R.O. water. The pellet was resuspended to a concentration of 1g packed cells in 10ml of R.O. water (Appendix 1.2), aliquoted into 5-10ml volumes and stored at 4°C for up to 12 weeks. The purity of the suspension was checked by plate culture on blood agar (Appendix 1.6).

2.2 Live *Flexibacter maritimus* suspension

Marine Ordal's medium	1 litre
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(Appendix 1.7)

F. maritimus cultured on Marine Ordal's agar plates (Appendix 1.7)

Flexibacter maritimus was grown in one litre volumes of Marine Ordal's medium according to the method described for the culture of *E.coli* and *Stenotrophomonas maltophilia* (Appendix 2.1). Harvested cells were washed and resuspended with sterile sea-water (Appendix 1.1) instead of sterile R.O. water. Purity of the suspension was checked by plate culture on Marine Ordal's agar (Appendix 1.7).

2.3 Live marine gill flora suspension

Nutrient Broth No. 2 (Oxoid, code CM67)	25g
Sodium chloride	15g
Sucrose	10g
Yeast extract (Oxoid, L21)	1g
Natural sea-water	1 litre

Normal gill flora collected from primary amoebae isolations
(Method 2.2.1) on malt yeast agar plates
(Appendix 1.3)

All the ingredients except the bacterial cultures were mixed and then autoclaved at 121°C for 20 minutes. The medium was then cooled to room temperature and stored at 4°C for up to 8 weeks before use. Normal gill flora were grown in one litre volumes of this medium in a two litre flask. The flask was inoculated with as many distinct bacterial colonies present on at least 3 primary amoebae isolation plates (See Chapter 2, method 2) not older than 7 days post isolation. The medium was incubated at 20°C and processed as for *E.coli* and *St. maltophilia* (Appendix 2.1), using sterile sea-water (Appendix 1.1) instead of R.O. water.

3. Amoeba isolation reagents

3.1 Antibiotic cover 1

Streptomycin sulphate (Sigma, S6501)	0.1g
Benzylpenicillin G (CSL)	0.1g
Carbenicillin (Sigma, C1389)	0.1g
Ampicillin (Sigma, A9518)	0.25g
R.O. water	10ml

The antibiotics were mixed, filter sterilised with a 0.22µm filter cartridge, and then stored frozen at -20°C.

3.2 Antibiotic cover 2.

To 10ml of R.O. water 0.1g of erythromycin (Sigma, E6376) was added, the solution was then filter sterilised with a 0.22µm filter cartridge and stored frozen at -20°C.

3.3 Ammonium chloride gill wash solution (Jones, 1988)

A 2.5% ammonium chloride solution in natural sea-water was prepared and autoclaved for 15-20 minutes at 121°C. Immediately before use antibiotic cover 1 (Appendix 3.1) and 2 (Appendix 3.2), were added to the ammonium chloride solution at a ratio of 1:1000. The volume of solution prepared was dependent on the size and number of gills to be washed (Method 2.2.1)

4. Parasome staining techniques

4.1 Agar slides

Ethanol cleaned slides were coated with a 2% solution of non-nutrient agar (Difco Laboratories, 0140-01) in natural sea-water and allowed to dry in a vertical position. The slides were stored at 4°C in an airtight container

4.2 DAPI stock solution (Rogerson, 1988)

4'6-diamidino-2-phenylindole (Sigma D9542)	1mg
R.O. water	20ml

The ingredients were mixed and the solution stored at 4°C in the dark for up to 6 months.

4.3 Chrom gelatin slides (L. Barber, Pers. Comm.)

Gelatin	5g
Potassium dichromate	0.5g
R.O. Water	100ml

All the ingredients were mixed and then warmed to dissolve the gelatin. Ethanol clean slides were dipped in the warm solution and allowed to dry in a vertical position. Once dry the slides were stored at room temperature for a maximum of 7 days.

4.4 Schaudinn's solution (Spencer and Munroe, 1961)

Mercuric chloride, saturated aqueous	200ml
Ethyl alcohol, 95%	100ml
Glacial acetic acid	15ml

The mercuric chloride and ethyl alcohol were mixed and stored indefinitely at room temperature in a sealed bottle. Prior to use the glacial acetic acid was added to activate the solution, activated solution was discarded after use.

4.5 Gomori's trichrome stain (Spencer and Munroe, 1961)

Chromotroph 2R*	0.6g
Light green SF*	0.3g
Phosphotungstic acid	0.7g
Acetic acid	1.0ml
R.O. water	100ml

All ingredients except R.O. water were mixed and allowed to stand for 30-60 minutes, after which the R.O. water was added. After each use the stain was allowed to stand uncovered for at least 4 hours to allow excess alcohol to evaporate. The stain was stored at room temperature indefinitely.

* Manufactured by National Aniline Division, Allied Chemical and Dye Corporation, New York

5. Estimation of cell number by microscopy

Total count

The total number of amoebae, viable and non-viable present in a suspension was estimated using a haemocytometer. Amoebae were harvested from plate cultures by flooding the culture plate with filtered sea-water and dislodging the cells from the surface using a sterile spreader. The suspension was then thoroughly mixed to prevent clumping and a drop placed onto the surface of the haemocytometer. The number of amoebae were calculated by counting the mean number of cells counted across four individual squares of the haemocytometer, and applying a standard formula. Small squares were counted if the density of cells was high, and large squares if the density was low. The formulae for the haemocytometer used in this study were as follows:

Formula for large squares

$$P \times 9 \text{ (no of large cells)} \times 1.1 \times 1000 = \text{no. of amoebae/ml}$$

Formula for small squares

$$P \times 16 \text{ (no of small squares)} \times 9 \text{ (no of large squares)} \times 1.1 \times 1000 = \text{no. of amoebae/ml}$$

(P=number of cells)

6. Gill tissue fixatives

6.1 Sea-water formalin (adapted from Handler and Clark, 1990)

Formalin

Natural sea-water, 0.45µm pre-filtered (Appendix 1.1)

Formalin was diluted to 10% in natural sea-water and stored at room temperature.

6.2 Isotonic formalin (Handler and Clark, 1990)

Formalin

R.O. water

Formalin was diluted to 10% in R.O. water and stored at room temperature.

6.3 Sea-water Davidson's (adapted from Handler and Clark, 1990)

Ethanol 95%	300ml
Natural sea-water, 0.45µm pre-filtered (Appendix 1.1)	300ml
formalin	200ml
glacial acetic acid	100ml

All ingredients except glacial acetic acid were mixed together and stored at room temperature. The glacial acetic acid was added less than 48 hours prior to use, and the precipitate allowed to settle. Activated Sea-water Davidson's was discarded after 48 hours.

6.4 Isotonic Davidson's (Handler and Clark, 1990)

Ethanol 95%	300ml
R.O. water	300ml
Formalin	200ml
Glacial acetic acid	100ml

All ingredients except glacial acetic acid were mixed together and stored at room temperature. The glacial acetic acid was added less than 48 hours prior to use.

'Activated' Isotonic Davidson's was discarded after 48 hours.

6.5 Bouin's (Clarke, 1973)

Saturated aqueous picric acid solution	750ml
Formalin	250ml
Glacial acetic acid	50ml

All ingredients except glacial acetic acid were mixed together and stored at room temperature. The glacial acetic acid was added less than 48 hours prior to use.

'Activated' Bouin's was discarded after 48 hours.

7. Preparation of tissue sections for immunostaining

7.1 Poly-L-Lysine® coated slides (Marchant and Thomas, 1983)

Slides cleaned with acid alcohol (1% v/v HCl in 70% alcohol) were allowed to soaked in Poly-L-Lysine (Sigma, P8920) diluted 1:10 in R.O. water, for 5 minutes. Slides were dried at 60°C for 1 hour and then stored in a sealed container for up to 4 weeks. The diluted Poly-L-Lysine solution was then filtered through at 0.45µm filter and stored at 4°C for 3-4 months, unless visibly contaminated.

7.2 Preparation of paraffin embedded fixed tissues

(DPIWE method)

Sections of paraffin embedded tissues were cut approximately 5-10µm thick and dried onto Poly-L-Lysine slides (Appendix 7.1). The slides were then placed in X3B solvent for 5-10 minutes to remove the wax. Sections were brought to water by rinsing in 100% ethanol, then soaking in 70% ethanol for 3-5 minutes and finally rinsing for 5 minutes in a continuous flow of tap water. Sections were immediately immunostained.

8. Immunostaining Reagents

8.1 Positive control smears

Positive control smears for both the IFAT and field assays were prepared from fish with advanced AGD, that had more than 10 mucus patches per fish. Fish were euthanased with an overdose of benzocaine (70-100mg/L). Gill arches were excised, and one arch placed in sea-water Davidson's fixative (Appendix 6.3) to confirm its AGD status by histological examination. Material on the remaining gill arches was smeared across clean microscope slides, at a ratio of two slides per gill arch. The smears were then air dried and stored at 4°C.

8.2 Phosphate buffered saline (PBS) (DPIWE recipe)

R.O. water	20 litres
Na ₂ HPO ₄	14.48g
KH ₂ PO ₄	4.48g
NaCl	153g

The chemicals were dissolved in 1 litre of R.O. water before being made up to 20 litres. The pH was checked and corrected to pH 7.2 if required, and the buffer stored at room temperature.

8.3 IF (immunofluorescence) antibody diluent

(Polack and Van Noorden, 1984)

PBS pH 7.2 (Appendix 8.2)

Bovine serum albumin (BSA) crystalline-grade (CSL, 06711701)	0.1% (w/v)
Sodium azide	0.1% (w/v)

All ingredients were mixed and stored at 4°C. Buffer was discarded if visibly contaminated.

8.4 Alkaline buffered glycerol (DPIWE recipe)

NaHCO ₃	0.0729g
Na ₂ CO ₃	0.016g
R.O. water	10.0ml
Glycerol	90.0ml

The salts were dissolved in the distilled water before adding the glycerol, the pH was adjusted to pH 9.0 if necessary. The buffer was stored at 4°C indefinitely unless visibly contaminated.

8.5 IP (immunoperoxidase) antibody diluent

(Polack and Van Noorden, 1984)

PBS pH 7.2 (Appendix 9.2)

Bovine serum albumin (BSA) crystalline-grade (CSL, 06711701) 0.1% (w/v)

The BSA and PBS were mixed and stored 4°C for no more than 2 weeks.

8.6 DAB peroxidase substrate (DPIWE method)

Hydrogen peroxide (made in distilled water from 30% stock)	0.02%(w/v)
Diaminobenzidine tetrahydrochloride, DAB (made in 0.1M Tris Buffer, pH 7.2)	0.1% (1mg/ml)

Mix together an equal volume of each just prior to use. The hydrogen peroxide should be freshly prepared from concentrated stock. DAB can be made up in advance and frozen in aliquots.

8.7 Alkaline phosphatase (AP) substrate buffer

(Vectastain ABC kit product information, Vector Laboratories, USA)

Tris-HCl pH 8.2	100mM
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Levamisole (Sigma, L9756)	1mM
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Ingredients were mixed and stored at 4°C indefinitely, unless visibly contaminated.

8.8 Counterstaining with haematoxylin

Immunostained gill sections were counterstained with haematoxylin (Appendix 8.9) for 60 seconds followed by a 10 second rinse in 70% ethanol, 20 seconds in 100% ethanol, a further 20 second rinse in 100% ethanol and finally in X₃B for 60 seconds prior to mounting with D.P.X (BDH product no. 36029).

8.9 Haematoxylin (Mayers)

(Bancroft and Stevens, 1975)

Haematoxylin	1g
R.O. water	1000ml
Potassium alum	50g
Citric acid	1g
Chloral hydrate	50g
Sodium iodate	200mg

The haematoxylin, potassium alum and sodium iodate were dissolved into warmed R.O. water, then the chloral hydrate and citric acid were added. The solution was then boiled for 5 minutes, allowed to cool, filtered, and left to cure overnight.

9. Cryopreservation media for amoebae

9.1 Freshwater and euryhaline cryopreservation medium

(Robinson *et al*, 1990)

Base

Proteose peptone No. 3 (Difco Laboratories, 0122-01-2)	10g
Yeast extract (Oxoid, L21)	0.5g
Folic acid	1g
Na citrate.2H ₂ O	0.5g
R.O. water	450ml

Inorganic ion supplements

1. 0.4M MgSO ₄	5ml
2. 0.05M CaCl ₂	4ml
3. 0.005M Fe(NH ₄) ₂ (SO ₄) ₂ .6H ₂ O	5ml
4. 0.25M Na ₂ HPO ₄ .7H ₂ O	5ml
5. 0.25M KH ₂ PO ₄	5ml

Organic supplements

2M Glucose (autoclaved at 121°C for 15 minutes)	25ml
Antibiotic Stock 1 (Appendix 3.1)	1.0ml
Antibiotic Stock 2 (Appendix 3.2)	1.0ml
DMSO (dimethyl sulphoxide)	50ml

The base ingredients were combined, the inorganic supplements were added in the order 1 to 5 to avoid precipitation. The pH was then checked and adjusted to 6.5 if necessary. The medium was then autoclaved at 121°C for 25 minutes and allowed to cool. After cooling the sterile glucose, antibiotic stocks and DMSO were added, the medium aliquoted and stored at 4°C. Sterility of the medium was checked by incubating a 10 ml aliquot at 25°C for 7 days and observing for growth.

9.2 Marine cryopreservation medium

(adapted from Robinson *et al*, 1990)

This medium was adapted for the cryopreservation of marine amoebae, it was made in the same manner as the freshwater cryopreservation medium (Appendix 9.1) except substituting the R.O. water with natural sea-water and reducing the amount of folic acid to 0.5g. Some precipitation of the folic acid was observed, this precipitate was allowed to settle before the medium was aliquoted and used, and did not appear to affect cryopreservation.

10. Reagents for electron microscopy staining (Jones, 1985)

10.1 Sodium cacodylate buffered sea-water

Sodium cacodylate 0.1M in R.O. water

Natural sea-water

A 0.1M solution of sodium cacodylate was mixed with an equal volume of natural sea-water. The solution was stored at 4°C indefinitely.

10.2 Glutaraldehyde - cacodylate fixative

Glutaraldehyde 3% (v/v)

Sodium cacodylate buffered sea-water (Appendix 10.1)

Glutaraldehyde was added to sodium cacodylate buffered sea-water to give a final concentration of 3% v/v. Solution was stored at room temperature indefinitely.

10.3 Osmium tetroxide

Osmium tetroxide (OsO₄) 1% (w/v)

Sodium cacodylate 0.1M in R.O. water

Osmium tetroxide was added to 0.1M sodium cacodylate to give a final concentration of 1% v/v.

10.4 Uranyl acetate

A saturated solution of uranyl acetate was prepared by adding 5-6g of uranyl acetate per 100ml of glass distilled water.

11. Reagents for *Paramoeba* ELISA

(Adapted from the principals discussed in Kenney, 1991)

11.1 Coating buffer

Na ₂ CO ₃	1.5g
NaHCO ₃	2.93g
R.O. water to 1 litre	

All ingredients were mixed, the buffer adjusted to pH to 9.6 if necessary and stored at 4°C.

11.2 Blocking buffer

PBS pH 7.2 (Appendix 8.2)

Tween 20	0.5% (v/v)
Casein	3% (v/v)

The casein and Tween 20 were added to the PBS and allowed to dissolve. The buffer was stored at 4°C for a maximum of 3 days.

11.3 ELISA wash buffer (EWB)

PBS pH 7.2 (Appendix 8.2)

Tween 20	0.05% (v/v)
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The PBS and Tween 20 were mixed and the buffer stored at room temperature for up to 7 days.

11.4 Dilution buffer (DB)

PBS pH 7.2 (Appendix 8.2)

Tween 20 0.5% (v/v)

Casein 3% (v/v)

The casein and Tween 20 were added to the PBS and allowed to dissolve. The buffer was stored at 4°C for a maximum of 3 days.

11.5 TMB diluent - citrate phosphate buffer (DPIWE method)

0.1M Citric acid 24ml

0.2M Na₂HPO₄ 26ml

The two ingredients were mixed, made up to 100ml with R.O. water and stored at 4°C.

11.6 TMB substrate (DPIWE method)

One TMB tablet (Sigma: 3,3', 5,5'-Tetramethyl benzidine dihydrochloride tablets, 1mg, Cat No. T-3405) was added to 10ml of TMB diluent (Appendix 11.5) approximately 30 minutes before use. Immediately before use 2µl of 30%v/v hydrogen peroxide was added. Excess activated substrate was discarded immediately after use.

11.7 Stop buffer - 1N sulphuric acid

Stop solution was prepared by diluting concentrated sulphuric acid 1:35 with R.O. water. The solution was stored at room temperature.

12. Adsorption of Serum

A volume of *St. maltophilia* culture (Appendix 2.1) equal to the volume of serum to be adsorbed was pelleted and the supernatant discarded. The pellet was then resuspended in the serum and was incubate overnight on a rotator at 4°C. After incubation the serum was centrifuged at 12,000g for 3 minutes and the serum collected and stored at -20°C.

13. Mucus Extraction Reagents

13.1 Extraction buffer (Lumsden *et al*, 1993)

saline	0.85% (w/v)
NEM (N- ethylmaleimide)	2mM
EDTA (ethylenediaminetetra-acetic acid -disodium salt)	10mM
sodium azide	0.02% (w/v)
PMSF (phenylmethyl sulphonyl fluoride)	2mM

The NEM, EDTA and sodium azide was dissolved in the saline solution. The PMSF was dissolved in a small amount of alcohol, and added immediately before use, as PMSF degrades rapidly in water. The extraction cocktail was used immediately and excess discarded after use.

13.2 Dialysis buffer (Lumsden *et al*, 1993)

R.O. water	
PMSF	2mM
Sodium azide	0.02% (w/v)

The dialysis buffer was prepared as per Appendix 13.1.

13.3 EDTA-Saline (DPIWE method)

EDTA	0.5% (w/v)
Saline	0.85% (w/v)

The EDTA was dissolved in the normal saline and the medium filter sterilised to 0.22µm. The buffer was stored at 4°C.

14. Chemicals selected as potentially amoebicidal to *Paramoeba* sp. 'AGD'

Amprolium hydrochloride: is an anti-coccidial used in cattle, sheep, dogs and prophylactically in laying poultry. It belongs to the pyrimidine group of drugs structurally similar to thiamine, and acts as a thiamine antagonist. Absorption is thought to be good. (McDougald and Roberson, 1988)

Berberine: has been used in the treatment of hepatic amoebiasis and as an anti-malarial. It is one of the alkaloids present in hydrasis, in various species of *Berberis*, and many other plants. Mode of action is unknown. Absorption from the gut is good. (Dutta, 1981)

Benzylkonium chloride ('Zephiran'): is a cationic surfactant, detergent, made up of quaternary ammonium derivatives. It is used as a disinfectant for hatchery equipment and utensils, it has also been recommended for the control of bacterial gill disease in farmed Pacific salmon. The mode of action is thought to be due to the breakdown of various cellular enzymes and disorganisation of cell membranes. (Hoskins and Dalziel, 1984; Byrne *et al*, 1989; Alexander, 1991)

Carnidazole: is a 5-nitroimidazole derivative, similar to metronidazole. It is employed in veterinary practice in the treatment and control of trichomoniasis in birds. For action and absorption see metronidazole. (Martindale Pharmacopoeia, 1989)

Chloramine-T: sodium p-toluenesulphonchloramide ('Halamid', Akzo Chemicals) is an organic derivative of chlorine, with the bactericidal action and uses of chlorine. It has been employed as a wound disinfectant and general surgical antiseptic, and also as for the treatment of drinking water containing organic matter. It has been shown to be effective, as a bath treatment, for a wide range of micro-organisms affecting farmed fish, and in the disinfection of equipment used in hatcheries. (Information from AKZO Chemicals Ltd; Herwig, 1979; Martindale Pharmacopoeia, 1989)

Chloroquine diphosphate: is widely used to treat hepatic amoebiasis but is mainly an anti-malarial. It belongs to the 4-aminoquinoline group of chemicals. Action is by binding to native DNA preventing transcription. Absorption from gut is rapid and complete. The drug is stored in the liver and tissue and is slowly excreted. (Knight, 1980; Dutta, 1981; Kovacs and Masur, 1987)

Diloxanide furoate: is used as an anti-amoebic compound in the treatment of luminal amoebiasis, and has been reported to have high amoebicidal activity *in vitro*. It is an amide, a dichloroacetanilide derivative, structurally similar to the antibiotic chloramphenicol. Action may be due to blocking protein synthesis, as with chloramphenicol. Absorption from gut is poor. (Knight, 1980; Dutta, 1981; Kovacs and Masur, 1987)

Emetine dihydrochloride: is an early anti-amoebic compound used to treat both amoebic dysentery and extraintestinal amoebiasis. Emetine is an alkaloid, extracted from the plant *Cephaelis ipecacuanha*. It inhibits protein and DNA synthesis. It is not readily absorbed from the gut. (Hawkins, 1973; Knight, 1980; Dutta, 1981; Neal, 1983).

Fumagillin: is an antibiotic produced by the fungus *Aspergillus fumigatus*. It has been reported to be highly effective in the treatment of the kidney parasite *Sphaerospora renicola* of the common carp *Cyprinus carpio*. Its efficacy in the treatment of other myxosporean infections in fish has also been reported (Wishovsky *et al*, 1990; Yokoyama *et al*, 1990). However some toxicity problems have been reported (Wishovsky *et al*, 1990; Lauren *et al*, 1988). It is a potent amoebicide *in vitro*, with lower activity in humans. Absorption from the gut thought to be good. Mode of action is unknown. (Dutta, 1981; Molnár *et al*, 1987; Molnár, 1993; Kent and Dawe, 1994; Higgins and Kent, 1996; le Gouvello *et al*, 1999; Speare *et al*, 1999)

5-hydroxy- 1,4 naphthoquinone ("Juglone") and 2-hydroxy-1,4 naphthoquinone: both compounds belong to the hydroxynaphthoquinone group, members of this group have shown have both anti-coccidial and anti-malarial activity. Action thought to be due to blocking anaerobic respiration. Absorption properties unknown. (Hudson *et al*, 1985; Brown and Green, 1987)

Hydrogen peroxide: is commonly used as an antiseptic, it is a recognised bacteriostat and is an effective sporicide. More recently, the successful use of hydrogen peroxide as a delousing treatment for farmed Atlantic salmon infected with the sea-louse (*Lepeophtheirus salmonis*) has been described. In bacteria the mechanism for action is thought to occur as a result of multiple cellular injuries, or in the case of spores the removal of the protein coat surrounding the spore. In salmon louse it is postulated that bubbles form within the body of the sea-lice, causing paralysis. (Baldry, 1983; Bruno, 1992a; Johnson *et al*, 1993a)

8-hydroxyquinoline and 5-chloro-7-iodo-8-hydroxyquinoline : both compounds belong to the quinoline group. They have been widely used as to treat amoebic dysentery. Action is to chelate ferrous ions. Absorption is very poor. (Hawkins, 1973; Knight, 1980; Dutta, 1981;)

Ivermectin: belongs to a chemically related group of anthelmintics called the avermectins, chemicals of this group are produced by the fermentation of an actinomycete, *Streptomyces avermitilis*. Action is to inhibit the motility of the parasite by increasing the release of γ -aminobutyric acid inhibiting neurotransmission and muscle contraction. Absorption from the gut is good. (Rew and Fetterer, 1987) Ivermectin has been used to treat sea-lice infections of sea-farmed salmonids in some countries (Johnson *et al*, 1993b)

Levamisole: belongs to the imidothiazole group of chemicals. It is used against a wide spectrum of nematodes in man and animals. It is also shown to act as an immunostimulant. Action is most likely due to paralysis of the nervous system of the nematode. It is rapidly absorbed from the gastrointestinal tract. (Marriner and Armour, 1987; Martindale Pharmacopoeia, 1989; Rew and Fetterer, 1987)

Mebendazole: is an anthelmintic commonly used in deworming dogs, sheep and horses. Has also been used in the treatment of *Pseudodactylogyrus sp.* infecting farmed eels. It belongs to the benzimidazole group of chemicals. Precise mode of action is unknown however it is thought to be by inhibiting glucose uptake interfering with respiration and nutrition of the worms. Absorption from gut is rapid. (Arundel, 1987; Buchman and Bjerregaard, 1990)

Mefloquine hydrochloride ('Lariam', Roche): is an anti-malarial drug, reserved for multiple drug-resistant cases of malaria. It belongs to the 4-quinoline-methanol group of drugs, chemically related to quinine. Absorption from gut is variable. (Geary and Jensen, 1987)

Metronidazole and tinidazole: are used in the treatment of amoebic dysentery, hepatic amoebiasis, giardiasis, trichomoniasis and has a broad spectrum of reactivity against a wide range of anaerobic bacteria and protozoa. They belong to the nitroimidazole group of drugs. Action is thought to be due to inhibition of anaerobic respiration, or by intercalating with DNA preventing DNA and RNA synthesis. Absorption is rapid and complete (Hawkins, 1973; Lindmark and Muller, 1976; Knight, 1980; Dutta, 1981; Neal, 1983; Kovacs and Masur, 1987; Looker *et al*, 1987; McDougald and Roberson, 1988)

Monensin and narasin: are members of the ionophore group of compounds. They are active against the trophozoite form of *Coccidia* species, rendering the membranes permeable to sodium and potassium ions. They are poorly absorbed from the gut. (Macqueen, 1987; McDougald and Roberson, 1988)

Naphthalophos: is an organophosphorus compound that has been used as a veterinary anthelmintic. (Martindale Pharmacopoeia, 1989)

Nifurpirinol (Prefuran): is a nitrofur used primarily as an external treatment for fin rot, bacterial gill disease and external protozoan parasites. The drug has also been given orally to treat bacterial diseases in salmonids, caused by *Aeromonas* and *Vibrio* species, and hexamitiasis in Siamese fighting fish. Absorption properties are unknown. Nitrofurans form free radicals that reduce molecular oxygen. (Herwig, 1979; Ferguson and Moccia, 1980; Sedgewick, 1988; Looker *et al*, 1987;)

Niridazole: is used as a schistosomicide and as a potent amoebicide, and against axenic *E. histolytica in vitro*, and also in the treatment of protozoan and monogenean infections of fish. It belongs to the nitroheterocycle group of compounds; a nitrothiazole derivative, related to the nitroimidazoles. Action is not known but may be similar to the nitroimidazoles (see metronidazole). Drug is slowly absorbed from the gut and rapidly metabolised. (Knight, 1980; Dutta, 1981; Neal, 1983; Martindale Pharmacopoeia, 1989; Tojo *et al*, 1993, 1994)

Nitrothiazole: is a nitroheterocycle compound. Drugs from this group such as acinitrazole and tenonitrozole have been used to treat trichomoniasis in humans. Absorption is thought to be good. (Neal, 1983; Martindale Pharmacopoeia, 1989)

Nitrofurantoin: It is a nitroheterocycle compound, a synthetic antibacterial, a nitrofur derivative. Its action may be due to the formation of toxic hydroxyl radicals that cause peroxidation of DNA and lipids. Alternatively it may interfere with several bacterial enzyme systems. It is used to treat urinary tract infections in humans and is well absorbed when given orally. (Looker *et al*, 1987; Neal, 1983; Martindale Pharmacopoeia, 1989)

4-Nitroimidazole: belongs to the nitroimidazole group of drugs, such as metronidazole and tinidazole. It has a broad spectrum of activity against a wide range of anaerobic bacteria and protozoa. The action of nitroimidazoles is thought to be due to the drug interacting with the nucleic acids of the parasite. Absorption is thought to be good. (Neal, 1983; McDougald and Roberson, 1988)

Oxfendazole: is one of the most active benzimidazoles used to treat nematode and cestode infections in sheep. Action and absorption is as for mebendazole (Arundel, 1987)

Ozone: has been reported as an effective bactericide and protozoicide, its use as a disinfectant for freshwater aquaculture systems has also been recognised. Ozone molecules are highly unstable and readily breakdown to form one oxygen and one highly reactive oxygen free radical, the biocidal properties of ozone relate to the high oxidising potential of this free radical. (Williams *et al*, 1982; Korich *et al*, 1990; Sugita *et al*, 1992)

Phthalylsulphathiazole and sulphaquinoxaline: both belong to the sulphonamide group of chemicals. Compounds of this group are used widely in the treatment of coccidiosis. Action is due to competitive inhibition with p-amino benzoic acid (PABA). Absorption is rapid and complete. (McDougald, 1987; McDougald and Roberson, 1988)

Praziquantel: is a broad spectrum anthelmintic used in the treatment of worm infestations in a range of animals. It has been used to treat monogenean worm infestations in pond reared fish. Praziquantel is an pyrazinoisoquinoline derivative. Its action is by causing paralysis in susceptible worms, and damaging their tegument. Absorption is good. (Schmahl and Mehlhorn, 1985; Harnett, 1988)

Primaquine diphosphate: is used as a radical cure of resistant strains of malarial parasites, *Plasmodium* species. It belongs to the 8-aminoquinoline group. Action is unknown. It is readily absorbed from the gut. (Geary and Jensen, 1987; Martindale Pharmacopoeia, 1989)

Pyrantel: is used to treat threadworm, roundworm and hookworm. Acts as a neuromuscular blocking agent causing paralysis. It is highly absorbed from the gut. (Arundel, 1987; Botero, 1987)

Pyrimethamine: is used as both an anti-malarial and anti-toxoplasmosis drug. It belongs to the diaminopyrimidine group of drugs. Its action is to disrupt protein synthesis and nuclear division by blocking the action of the enzyme dihydrofolate reductase. Absorption is rapid. (Geary and Jensen, 1987; Looker *et al*, 1987)

Quinacrine hydrochloride: has been used in the treatment of giardiasis, coccidiosis and malarial infections. It has been suggested as a possible treatment for a wide range of fish skin protozoa and in the treatment of ciliate infestation in prawns. It is highly absorbed and widely distributed in the tissues and body, accumulating in the liver, spleen, lungs and adrenal gland. Action is thought to be due to intercalating into the DNA, inhibiting DNA and/or RNA synthesis. (Herwig, 1979; McDougald and Roberson, 1988)

Quinine hydrochloride: is an early anti-malarial drug. It is the main alkaloid in the bark of the Cinchona tree. It acts by intercalating with the DNA. Absorption is good. (Geary and Jensen, 1987)

Quinoline: is used prophylactically in chickens to control coccidia. Acts by disrupting electron transport in the mitochondrial cytochrome system. Absorption from gut unknown. (McDougald and Roberson, 1988)

‘Toltrazuril’ (Bayer): is a member of a new group of compounds, the symmetric triazinones. It has been found to be a highly effective coccidiostat in birds and mammals. Recently it has been shown to be active against fish coccidia, microsporidia, myxozoa and monogenean worms. There is no known biochemical mode of action, but it results in vacuolisation and lysis of the parasitic tegument. Absorption into fish cells has been demonstrated. (Mehlhorn *et al*, 1988; Schmahl and Mehlhorn, 1988; Schmahl *et al*, 1988 and 1989a, b, c & d)

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